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SYMPOSIUM
ON
ANTIBODIES:
THEIR PRODUCTION AND MECHANISM OF ACTION

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THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA
1957

SYMPOSIUM

ON

ANTIBODIES: THEIR PRODUCTION AND MECHANISM OF ACTION

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April 8 – 10, 1957

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INTRODUCTION

A research conference on ‘‘Antibodies: Their Production and Mechanism of Action’’ was held at Gatlinburg, Tennessee, April 8–10, 1957. This, the tenth in a series of conferences sponsored by the Biology Division of the Oak Ridge National Laboratory and the Division of Biology and Medicine of the Atomic Energy Commission, represented an important step in the development of the study of radiation effects. It provided an opportunity for discussion of a promising method of encouraging radiation recovery that, only a few years ago, was not available. A good part of the Biology Division activities have always included the use of certain agents for modifying radiation damage. We thought it wise not to concentrate all the discussion on stimulation of radiation recovery by bone marrow but also to review the immunological background for the study of bone marrow transplantation in mammals. This problem, although still in its early development, was discussed because of its great promise in producing recovery from radiation damage and also in pathology and immunogenetics. The genetic implications of this year’s topic form an important facet in the foundation for the 1958 symposium on ‘‘Genetic Approaches to Somatic Cell Variation.’’ Open discussions were encouraged; some of these are reproduced in this volume.

This series of symposia, started in 1948, have covered a wide range of subjects, all of which have been of great importance to the work conducted in the Biology Division and have broadened our horizon in the study of radiation effects.

Dr. T. Makinodan and Dr. C. C. Congdon had a major part in the arrangement of the program and also served as consultants to the editor. Their help is gratefully acknowledged. Dr. W. H. Taliaferro and colleagues were most helpful in the early development of this symposium.

Previous symposia in this series are:

1948 — Radiation Genetics
1949 — Radiation Microbiology and Biochemistry
1950 — Biochemistry of Nucleic Acids
1951 — Physiological Effects of Radiation at the Cellular Level
1952 — Some Aspects of Microbial Metabolism
1953 — Effects of Radiation and Other Deleterious Agents on Embryonic Development
1954 — Genetic Recombination
1955 — Structure of Enzymes and Proteins
1956 — Biocolloids

ALEXANDER HOLLAENDER

GENERAL INTRODUCTION: SYNTHESIS AND DEGRADATION OF ANTIBODY

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EIGHT FIGURES

The scanty knowledge of the mechanisms of synthesis and degradation of antibody is reviewed as an appropriate introduction to the wide range of interests covered in this symposium. As data accumulate, most of them fall in line with the findings on other proteins in mammals and microorganisms. These similarities will be stressed.

STABILITY OF THE ANTIBODY MOLECULE

After antibody appears in the serum, it undergoes a constant degradation into its component amino acids, which in turn become available for the synthesis of more antibody and of other proteins. Thus, at the organismal level, the synthesis and degradation of antibody are a part of the dynamic equilibrium between anabolism and catabolism of body proteins. In marked contrast, as long as the antibody molecule retains its specific immunological reactivity, it is chemically stable to the extent that its amino acids and certain of its constituent atoms are not in a dynamic state with similar amino acids or atoms in other compounds of the host. The first of these characteristics is important in the dynamics of the rise and fall of antibody during immunization and the second one is vital in interpreting some of the data obtained with isotopically labeled antibody.

Metabolic degradation. In general, the biological half life of antibodies in small animals with high metabolic rates, such as the mouse, is shorter than in larger animals with low metabolic rates, such as the rabbit. Furthermore, increasing the metabolic rate by the use of thyroxin decreases the half life. Metabolic rate is not the sole factor involved since the half life of antibody in children, with their higher rate of metabolism, is longer than in adults (Dixon *et al.*, '52). Nevertheless, the half life in a given species is remarkably constant under ordinary laboratory conditions, as indicated by the small standard errors obtained in the passive transfer of antibodies in nonimmunized rabbits (Taliaferro and Talmage, '56, and table 1).

The biological half life of antibody is probably most accurately determined by ascertaining the rate of disappearance of passively transferred antibody from the blood of a nonimmunized recipient by immunological methods. Approximate determinations of antibody half life can be made by iodinating the antibody with I^{131} and following the total radioactivity per milliliter of serum or per gram of blood in passively immunized recipients (Dixon *et al.*, '52; Taliaferro and Talmage, '56). This method obviates the necessity of titrating antibody and has the advantage that only small samples of blood need be taken from the experimental animal. The accuracy of this method, however, depends fundamentally on the purity of the iodinated antibody used for passive transfer or, conversely, on the similarity of the decay rate of the antibody and the contaminating iodinated serum proteins accompanying the antibody. When the globulin fraction of the serum was iodinated and the half life of globulin and antibodies of various animals were similar, as in the early study of Dixon *et al.*, ('52), contaminating serum proteins introduced very little error. Such contaminations introduce serious errors in the case of rabbit hemolysins because of the short half life of the antibody (Taliaferro and Talmage, '56). Nevertheless, I^{131} labeling gave excellent results when the labeled antibody used for passive transfer was purified by specific absorption on parti-

TABLE 1

Forssman antisheep red cell antibodies in the rabbit

	Data from:		
Characteristics	Stelos and Talmage ('57)	Highly hemolytic	Weakly hemolytic
Molecular weight	Stelos ('56); Talmage *et al.* ('56b)	900,000	165,000
Rate of hemolysis	R. S. Weinrach, M. Lai, and D. W. Talmage (unpublished data)	Varies as the square of the concentration	Varies as the 4th power of the concentration
Blood/tissue equilibration	Taliaferro and Talmage ('56)	82/18	50/50
Electrophoretic mobility	Stelos and Talmage ('57); Stelos ('56)	γ_1 (T fraction)	γ_2
Peak titer	Talmage *et al.* ('56a)	Early	Late
Half life	Taliaferro and Talmage ('56)	2.81 ± 0.12 days	5.56 ± 0.17 days

culate antigen or by precipitation with specific antigen and then elution with carrier antibody at neutral or acid *p*H or elution with acid alone. By such methods, Talmage *et al.* ('54) obtained eluates in which over 80% of the I^{131} in the eluate was coupled to antibody protein whereas in the original labeled globulin from which the eluate was prepared only about 3% of the radioactivity was antibody bound.

Determinations of the antibody half life in an actively immunized animal, based on the drop in percentage of isotope concentration of antibody after cessation of isotope administration, is accurate only when the degradation of antibody exactly balances the formation of antibody (the so-called steady state, such as holds for normal serum proteins) and when there is no appreciable production of labeled antibody caused by the recycling of labeled amino acids from the degradation of previously labeled antibody and other serum proteins. The first condition is difficult to attain and the second virtually impossible to achieve after administration of labeled amino acids to actively immunized animals. For example, the long half life of two weeks for rabbit pneumococcus antibody, reported in the early work of Schoenheimer *et al.* ('42), was probably caused by the observations being made while the total amount of antibody was decreasing.

The biological half life may differ in two antibodies formed simultaneously in the same animal. Thus, of the antibodies with Forssman specificity in rabbits immunized with sheep red cells, a small part, which can be adsorbed on red cells, is large (ca. 900,000 molecular weight), is highly hemolytic, and has a half life of about 2.8 days. Most of the antibody, which can be adsorbed on red cells, is small (ca. 165,000 molecular weight), is only slightly hemolytic, and has a half life of about 5.6 days (table 1). Two antibodies with sheep isophile specificity also occur. In each specificity, the large and small molecules have similar, if not identical, immunological specificity as indicated by cross blocking (Taliaferro and Talmage, '56). Figure 1 shows the comparative half life of the four antibodies. It should be noted that the smaller antibody is titrated as the combining capacity (cbg) with red cells and hence includes both the large and small types. This procedure does not introduce an appreciable error because the serums studied contained very small percentages of the large type.

The speed of metabolic decay of the pneumococcus (type III) antibody is not affected by previous hyperimmunization (Humphrey and McFarlane, '54) or of hemolysin by a previous primary response (Taliaferro and Talmage, '56). These findings suggest that the presence of the antibody-synthesizing mechanism does not increase the speed of degradation.

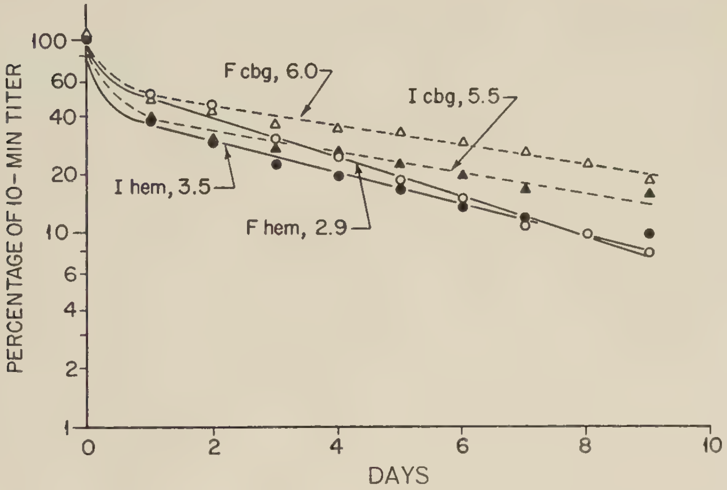

Fig. 1 Four antibodies formed in the same rabbit, showing different rates of decay when passively transferred to a normal rabbit. The individual half lives of the Forssman and isophile hemolytic activities (F hem and I hem) and of the Forssman and isophile combining activities (F cbg and I cbg) are shown by the numbers on each curve. (From Taliaferro and Talmage, '56.)

In marked contrast to antibodies, there is accumulating evidence that the synthesis of bacterial enzymes and probably of all bacterial proteins is virtually irreversible and that there is no interplay of protein degradation and synthesis except in whole cultures containing both dying and actively dividing cells (Monod and Cohn, '53; Rotman and Spiegelman, '54; Koch and Levy, '55; Hogness *et al.*, '56; Monod, '56). In comparing the bacterial system and mammalian tissues, Hogness *et al.* ('55) point out that the bacterial cells (*Escherichia coli*) used to demonstrate the irreversibility of protein synthesis constitute a homogeneous population of cells in an identical environment in which there is no observable cell lysis or secretion of proteins by the cells. They further point out that there are three possible pathways by which tissues may lose proteins, viz., by intracellular degradation, by secretion, and by cell lysis.

The conclusions of Monod and Spiegelman and their coworkers on the lack of intracellular protein degradation has been questioned by Steinberg *et al.* ('56); but, in any case, the degradation of intracellular proteins would seem to be much slower than that of antibody proteins. Furthermore, it should be noted that the rate of metabolic degradation in the proteins of mammals varies markedly. Serum proteins are among those that are degraded rapidly. In contrast, collagen is essentially metabolically inert (Neuberger *et al.,* '51; Thompson and Ballou, '56). Many of the relatively stable proteins can, however, be raided under certain conditions (Whipple, '56).

Dynamic states. The absence of a dynamic exchange of amino acids in and out of the intact antibody molecule was first shown by Heidelberger *et al.* ('42). These authors passively transferred carrier pneumococcus (type III) antibody to a recipient that was fed N^{15}-labeled glycine over a period of 50 hours before passive transfer. Under such conditions, the passively transferred antibody acquired a small amount of isotopic label. The same result was obtained when type I pneumococcus antibody was transferred to a rabbit actively forming type III antibody and being fed isotopically labeled glycine. Heidelberger *et al.* ('42) believed that the label was caused by nonantibody contaminants and therefore that there was no nitrogen exchange. This conclusion that the isotopic activity of antibody introduced into a labeled recipient represents ''nonspecific'' protein carried down by the antigen-antibody precipitates was confirmed by Bulman and Campbell ('53), although earlier a specific radioactivity by dynamic exchange was suggested (Kooyman and Campbell, '48). In addition, Taliaferro and Talmage ('55) passively transferred anti-BSA serum to a normal rabbit and then administered 13 injections of 0.1 mc of S^{35} in the form of yeast hydrolyzate at 12-hour intervals. Antibody removed from serum samples taken at 2, 5, and 7 days possessed a relatively low but significant radioactivity that could be accounted for by the binding of radioactive complement or other nonantibody materials.

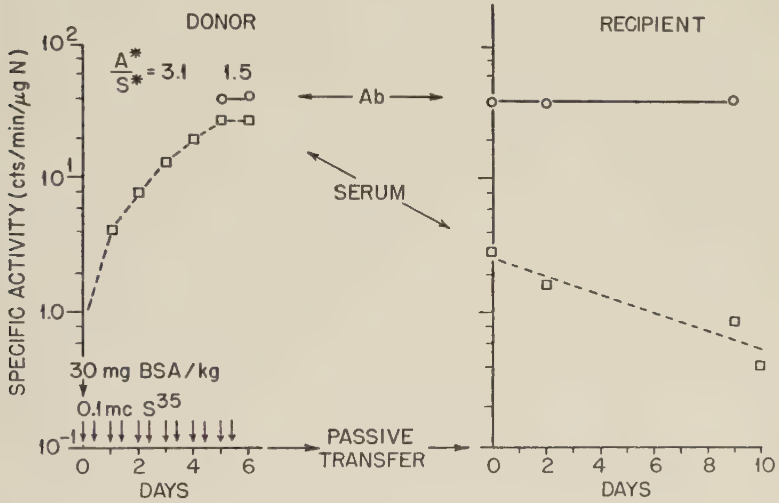

Fig. 2 The stability of the antibody molecule as shown by the constancy of the specific radioactivity in the immunized donor (secondary response to bovine serum albumin), which received S^{35}-labeled amino acids, and for 9 days after passive transfer in the recipient, which was not immunized and did not receive labeled amino acids. The behavior of the antibody is in marked contrast to that of the TCA-precipitable proteins of the serum. (From Taliaferro and Talmage, '55.)

The stability of the antibody molecule can be more precisely demonstrated by passively transferring highly labeled antibody to an unlabeled recipient and testing for loss of isotopic label. This procedure obviates possible errors caused by the large amount of labeled nonantibody protein when the recipient receives labeled amino acids. Thus Gros *et al.* ('52a) found that the specific radioactivity of antibody, formed in an actively immunized animal administered valine labeled with C^{14} in its isopropyl group, was not significantly reduced 6 days after passive transfer to a recipient that was neither immunized nor given labeled amino acids (see also Dubert *et al.*, '53). Humphrey and McFarlane ('54) carried out more extensive experiments with type III pneumococcus antibody labeled by administering lysine and phenylalanine containing C^{14} in their carboxyl groups. They found no change in specific radioactivity during the three weeks when this antibody was passively transferred to nonimmunized recipients. The same absence of amino acid interchange is shown in figure 2 from the work of Taliaferro and Talmage ('55) on S^{35}-labeled antibody to bovine serum albumin. Here the specific radioactivity of antibody acquired in the actively immunized donor rabbit, 40.6 cts/min/μg of antibody nitrogen, was essentially identical immediately after passive transfer and remained unchanged for 9 days in the recipient. These results are in marked contrast to the specific radioactivity of trichloroacetic acid-precipitable proteins in the serum whose count was immediately lowered after passive transfer by dilution and then constantly decreased owing to the replacement of labeled by nonlabeled protein.

A similar stability in the bacterial proteins has been emphasized by Hogness *et al.* ('55) and Monod ('56).

SYNTHESIS OF THE ANTIBODY MOLECULE

Although antibodies appear to be normal globulins so modified as to react specifically with their inducing antigens, they are synthesized *de novo* from the amino acid pool. They are not synthesized by modifying preexisting globulins or other proteins and are probably not synthesized from small polypeptide fragments of proteins. Moreover, there are probably no long-lived amino acid-containing percursors during synthesis, which takes at most 30 minutes and probably much less time.

Nitrogenous sources. Serum γ-globulins are not a unique source of antibodies. Thus isotopically labeled γ-globulin and albumin were obtained from a rabbit administered valine labeled with C^{14} in its isopropyl group. These two labeled serum proteins were then each given to a rabbit as a source of antibody label during immunization with pneumococcus type II. The antibodies formed in the two rabbits did not significantly differ in their specific radioactivity (Gros *et al.*, '52b; Dubert *et al.*, '53). Similarly, ovalbumin antibody did not acquire significant radioactivity within 72 hours when it was formed in a rabbit that had received C^{14}-labeled γ-globulin from an unimmunized rabbit (Askonas *et al.*, '56). Green and Anker ('54) studied the relative isotope concentration of (1) serum globulin, (2) serum albumin, (3) the amino acid pool, and (4) ovalbumin antibody after administering carboxyl-labeled C^{14}-glycine or an algal hydrolyzate containing amino acids labeled with D^2, N^{15}, and C^{14}. They concluded that serum globulin is not a direct precursor of antibody and that antibody is synthesized *de novo* from amino acids. In this work, the relative concentration of isotopic glycine in the amino acid pool was determined from urinary hippuric acid after the daily feeding of benzoic acid. A similar conclusion to the effect that the enzyme proteins of microorganisms are synthesized directly from amino acids has been reached by several groups of workers (Rotman and Spiegelman, '54; Hogness *et al.*, '55; Monod, '56).

The absence of long-lived amino acid precursors of antibody. Besides its intrinsic interest, the occurrence of long-lived amino acid precursors is particularly important in any study of the kinetics of antibody formation. If, for example, such precursors are formed during the induction period and incorporated into antibody later during the rise of serum antibody, the rise of serum antibody cannot be used as a direct and probably not as an indirect measure of antibody synthesis. Long-lived amino acid precursors were reported in the first study of this question (Green and Anker, '54) when isotopically labeled amino acids were administered to rabbits at various periods during a secondary response to ovalbumin. Thus, when amino acids labeled with C^{14}, N^{15}, and D^2 were given to a rabbit 10, 41, and 89 hours, respectively, after antigen injection, all three isotopes were found in the antibody removed from the rabbit on the fifth day. From these data, 31% of the protein of the antibody was calculated to represent amino acids drawn from the amino acid pool during the first 60 hours, i.e., the approximate duration of the induction period. The remainder was supposed to be synthesized during the succeeding 72 hours. Later work indicated that this conclusion was unwarranted (Taliaferro and Talmage, '55; Dixon *et al.,* '56) because the results could be explained on other grounds; viz., a recycling of the labeled amino acids and the inclusion of labeled coprecipitating antibody (formed prior to the precipitating antibody) and of labeled complement or other nonantibody proteins with the antigen-antibody precipitate.

That long-lived amino acid precursors are not present is indicated by the following experiment of Taliaferro and Talmage ('55), which circumvented the complexities arising in the work of Green and Anker. After S^{35}-labeled amino acids were administered to donor rabbits during the induction period of a secondary response to bovine serum albumin, the spleens of the rabbits were removed at about the end of the induction period, minced, and injected into unlabeled nonimmunized recipient rabbits. The antibody-forming mechanism was thus transferred from a labeled rabbit to an unlabeled recipient, and antibody was produced in an unlabeled medium. Under these conditions, the antibody was labeled to only a small degree (not over 1% and probably about 0.3%). Similar results were reported by Stavitsky ('56). In a further study by Taliaferro and Taliaferro ('57), the initial antibody found in the recipients was very small in amount and was highly labeled, but its specific radioactivity dropped at a rate approximately inverse to the rate of rise of recipient antibody (fig. 3).

Results in the foregoing transfer experiments indicate that antibody, except for a small initial amount, is made from the amino acids of the recipient and, therefore, contains only a very small percentage of amino acids taken from the pool during the induction period. The absolute percentage of such labeled amino acids is a function of the amount of antibody formed in the recipient. Furthermore, the results suggest that the induction period is a period during which the antibody-synthesizing mechanism is formed and is not a period during which amino acids are incorporated into precursors that later form part of the antibody. It might be objected that the transfer of splenic materials from donor to recipient

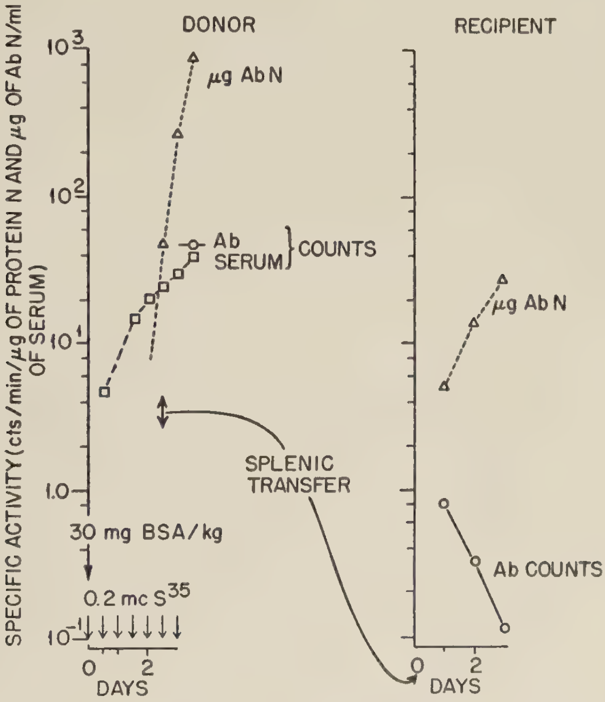

Fig. 3 A comparison of the specific radioactivity of antibody formed in a donor rabbit, which received S^{35}-labeled amino acids during a secondary response to bovine serum albumin, and of antibody formed in an unlabeled recipient, which received minced spleen from the donor 2.5 days after the initiation of the secondary response.

Note that the recipient antibody on the first day was 1.6% as radioactive as the donor antibody and dropped to 0.25% by the third day. The rate of decrease in the isotopic content of the recipient antibody was approximately the inverse of the rate of rise of serum antibody. (Taliaferro and Taliaferro, '57).

may have so damaged the antibody-synthesizing mechanism that the latter functioned abnormally in the recipient. Control experiments, however, were carried out in which the same amounts of S^{35}-labeled amino acids were given to the donor and to the recipient during the rise of serum antibody. Under such conditions, the same specific radioactivity occurred in donor and recipient antibody (Taliaferro and Talmage, '55).

Dixon *et al.* ('56) gave a single injection of S^{35}-labeled amino acids at various times during the first 7 days of the secondary response to bovine γ-globulin and ascertained the total radioactivity of antibody per milliliter of serum taken on the eighth day. Their plot of increasing radioactivity resembled the results of Green and Anker. When, however, they considered the incorporation of S^{35} on day zero as being caused by recycling, and subtracted it from subsequent values, the results were comparable to those of Taliaferro and Talmage.

INDUCTION OF ANTIBODY SYNTHESIS

In an over-all sense, antigen acts as the inducer for antibody synthesis, and the process is frequently assumed to be akin to the induction of the so-called adaptive enzymes in bacteria. As reasonable as this assumption is and as theoretically fruitful as it has been (Burnet and Fenner, '49), we should keep in mind that the mechanism of induction is as yet undefined and that it is not known whether the immediate inducer is all or only a part of the antigen. The absence of appreciable amounts of amino acid-containing precursors during the induction period strongly suggests that antigen primarily induces either the synthesis of the antibody-forming mechanism in the form of new cells or new intracellular enzymes or the formation of templates or other mechanisms to modify the already-existing, globulin-producing machinery to produce antibody. Such a sequential type of induction, even if it involves the production of new cells, does not preclude the synthesis of very small amounts of antibody shortly after the injection of antigen. Actually, in the secondary response from which the best data on the absence of appreciable quantities of precursors were obtained, the induction period is short enough to allow extrapolation of the logarithmic rise in serum antibody back to zero time and obtain reasonable values for beginning antibody synthesis. The assumption of a lag phase in antibody synthesis is more reasonable during the induction period of the primary response. Whether antibody synthesis is induced by antigen directly or indirectly through intermediate steps does not modify the conclusion reached previously that antibody formation represents the *de novo* synthesis of a new globulin from the amino acid pool.

Bacteria form a few molecules of an inducible enzyme in the absence of the inducer. The inducer is necessary for a greatly accelerated production of enzyme and, except in certain special cases, must continue to be present for the continued accelerated production. Some investigators believe that antigen also only induces a greatly accelerated production of an antibody that is already being made in small amounts before immunization.

The work on hemolysin production (Taliaferro and Taliaferro, '50, '51, '52) suggests that antigenic stimulation has to be present for marked antibody production. For example, the production of splenic antibody, after a single injection of antigen, rapidly declines when peak titer has been reached (see fig. 4). This decline is apparently caused by a lack of antigenic stimulation, since a second injection of antigen was followed by a second splenic response not significantly different from the first response. Another interesting feature of these experiments is that the spleen forms antibody at a high rate for a short time whereas nonsplenic sources form antibody at a slower rate for a much longer time (fig. 4). These results suggest that antigen or some antigen-induced product is exhausted in antibody formation. During continued antigen injection, however, the spleen and in time other sites eventually stop forming antibody in spite of continued antigen injections. Interpretation of this type of refractory state is difficult because the repeated injection of antigen may itself eventually inhibit the synthesizing mechanism (paralysis of the immune mechanism as suggested by Felton, '49) or increased amounts and more-avid serum antibody may inhibit the antigenicity of the antigen (Barr *et al.*, '50; Mason *et al.*, '55; Talmage, Freter, and Thomson, '56). The physiological mechanism that limits antibody synthesis during long-continued immunization may account for the dilemma of the ineffectiveness of persisting antigen to stimulate antibody production such as is discussed by Dixon *et al.*, ('56).

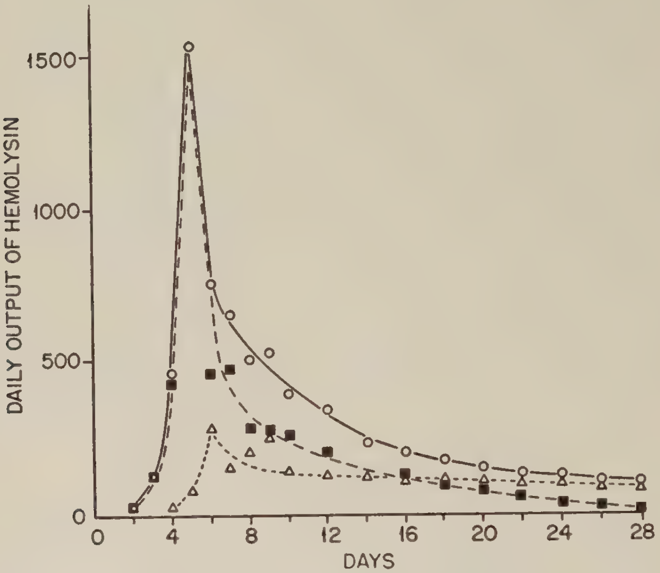

Fig. 4 Approximate mean total daily output of splenic and nonsplenic antibody as calculated from two groups of intact and splenectomized rabbits injected intravenously with 1 ml of 10% sheep red cells per kg and corrected for daily loss by metabolic degradation. ○, Splenic plus nonsplenic (intact); ■ - - -, splenic; △, nonsplenic (splenectomized).

Note that the rate of splenic antibody rose and fell precipitously whereas that of nonsplenic antibody rose to a lower peak and decreased less rapidly. (From Taliaferro, '55.)

Jaroslow and Taliaferro ('56) are probably defining some of the conditions necessary for induction even if not defining the inducing mechanism itself. Previous to their work, a marked suppression of antibody formation by total-body X irradiation was found to be caused by injury to a very early, possibly the initial, event in antibody formation (Kohn, '51, and especially Dixon, Talmage, and Maurer, '52; Taliaferro *et al.*, '52; Taliaferro and Taliaferro, '54). The early event may be almost instantaneous in duration and at most is only a few hours. Thus, as shown in figure 5, the power to form antibody after X-ray injury decreased 50% in about 2 hours. This decrease may be caused by the inhibition of the formation of some material necessary to initiate the antibody-

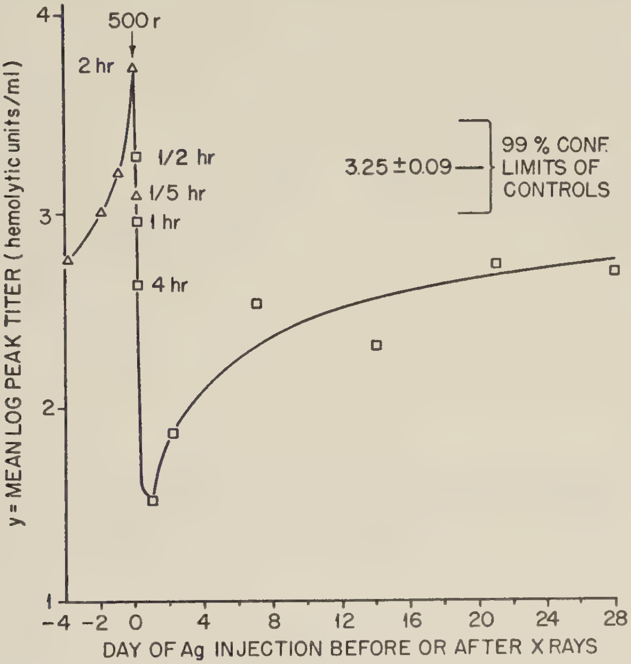

Fig. 5 Mean log peak hemolysin titer in 14 groups of rabbits given 0.125 ml of 1% sheep red cells per kg either before (5 groups) or after (9 groups) irradiation of the total body.

Note that mean log peak titer dropped precipitously starting with the group receiving antigen 2 hours before X irradiation (enhanced antibody production) and ending with the group given antigen 24 hours after irradiation. (From Taliaferro and Taliaferro, '54.) △, Before irradiation; □, after irradiation.

forming process. Experiments based on this premise demonstrated that yeast, HeLa cells and normal spleen contain a substance or substances that, when mixed with antigen, restore the hemolysin-forming capacity to rabbits made anergic by 400 r of total-body X radiation, as shown in figure 6

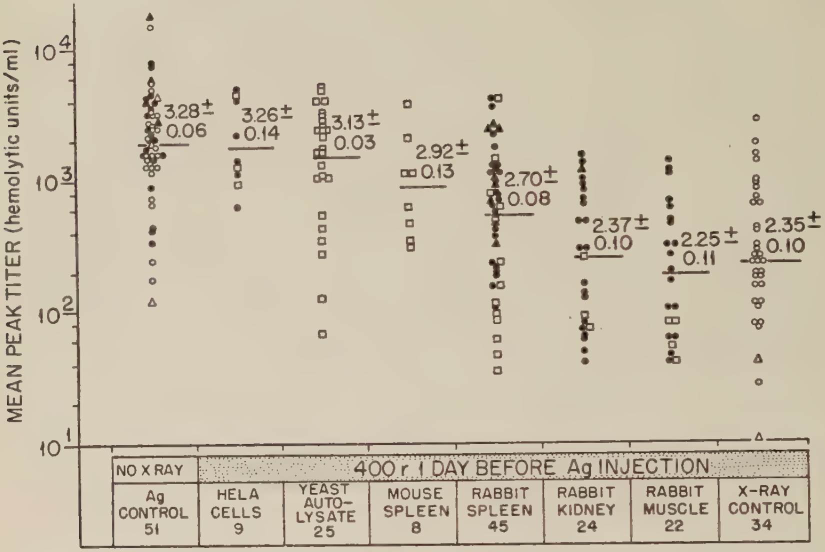

Fig. 6 Log peak hemolysin titers in six treated groups and in two control groups of rabbits (nonirradiated and irradiated).

Note that HeLa cells and yeast autolysate restored peak titer to the range of nonirradiated rabbits, whereas normal mouse or rabbit spleen minces or extracts only partially restored peak titer. The peak titer of rabbits receiving normal rabbit kidney or muscle mince were within the range of the irradiated control group. (From Jaroslow and Taliaferro, '56, with additional unpublished data.) Whole red cells: ○, no tissue; □, tissue extract; ●, tissue. Red cell stromata: △, no tissue; ▲, tissue mince.

(Jaroslow and Taliaferro, '56). Preliminary experiments further indicate that the restorative principle is a product of the action of the specific nuclease on ribonucleic or deoxyribonucleic acid.

The work of Jaroslow and Taliaferro may shed light on some of the recent studies of T. N. and S. Harris and of Šterzl and Hrubešová. Thus rabbits previously given 425 r of X rays to the total body formed antibody when injected with a mixture of antigenic materials from dysentery bacilli and cells from the popliteal and axillary lymph nodes, the spleen or blood leukocytes from unimmunized rabbits, whereas control X-rayed rabbits receiving only antigenic materials formed little if any antibody (Harris and Harris, '55; S. Harris *et al.,* '55, '56; T. N. Harris *et al.,* '55, '56). Somewhat similarly, 5-day-old rabbits (which did not respond to the antigen alone) formed antibody when injected with a fraction of splenic tissue from an immunized donor (Šterzl and Hrubešová, '56). This fraction consisted of nuclear and mitochondrial elements containing deoxyribonucleoprotein and ribonucleoprotein, respectively. It is possible that, in both sets of experiments, as in the work of Jaroslow and Taliaferro, a restorative factor overcame the nonresponsiveness of the X-rayed or young animals. It should be noted, however, that in the work of T. N. and S. Harris, there was no lengthening of the induction period in the X-rayed recipients, whereas Jaroslow and Taliaferro found that even with complete restoration of the hemolysin-forming capacity in X-rayed rabbit, the appearance of antibody in the serum was invariably greatly delayed. It should also be noted that Šterzl and Hrubešová do not believe that any antigenic material was carried over in their experiments.

Rate of antibody synthesis. The nearest approach to a measure of the time necessary to assemble amino acids into antibody is the so-called transit time or the time between administration of isotopes and appearance of the isotopic label in antibody. Obviously, this length of time includes not only the time necessary for the synthesis of antibody but also the time necessary for penetration of the amino acids into the cell, the passage of antibody from the cell and, *in vivo,* the passage of antibody from the site of synthesis to the blood. It is, therefore, of great interest that the transit time is closely similar in *in vivo* and *in vitro* work. Figure 7 shows the rate of incorporation of S^{35} into rabbit antibody during a secondary response to bovine serum albumin. In this case, the earliest significantly labeled antibody appeared 40 minutes after injection of the isotope (Taliaferro and Taliaferro, '57). Figure 8 from Askonas and White ('56), is strikingly similar. It shows the incorporation of C^{14} after the addition of C^{14}-glycine to preparations of sliced lymph nodes that were forming antibody *in vitro*. Similar transit times are reported from *in vitro* studies of the formation of albumin of the chicken (Peters, '53) and *in vivo* studies of serum proteins of the rabbit (Green and Anker, '54–'55) and pancreatic proteins of the rat (Junqueira *et al.*, '55). The transit time also gives an upper limit for the life of amino acid precursors or intermediates, but the actual time

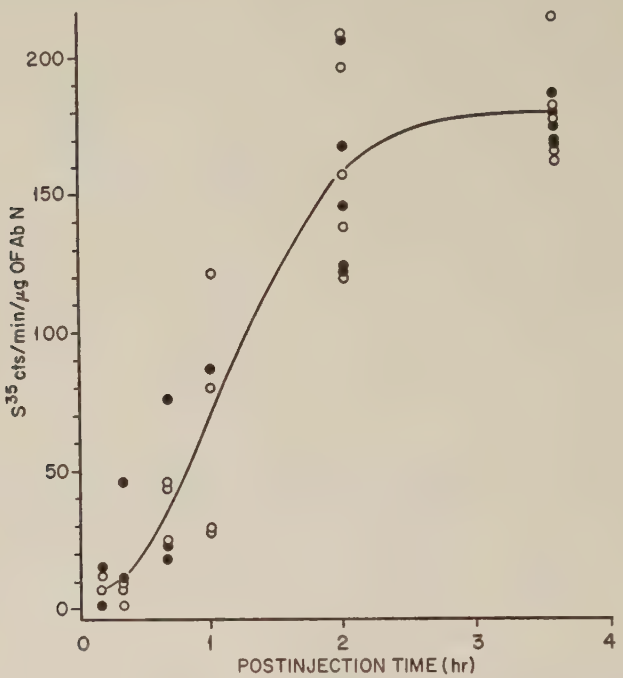

Fig. 7 The incorporation of S^{35} into antibovine serum albumin as shown by the rise in its specific radioactivity in rabbits during 4 hours. The animals were given one intravenous injection of 1 mc of S^{35}-labeled amino acids during the logarithmic rise of a secondary response. Solid circles, serums not decomplemented; open circles, decomplemented. (From Taliaferro and Taliaferro, '57.)

for antibody synthesis is probably much shorter. It may even approach the short interval of a few minutes as indicated for the synthesis of enzymes in bacteria (Hogness *et al.,* '55).

The previous discussion leads to the question of whether changes in serum antibody titers after correction for metabolic decay give a reasonable estimate of changes in the actual synthesis of antibody. When antigen is injected in-

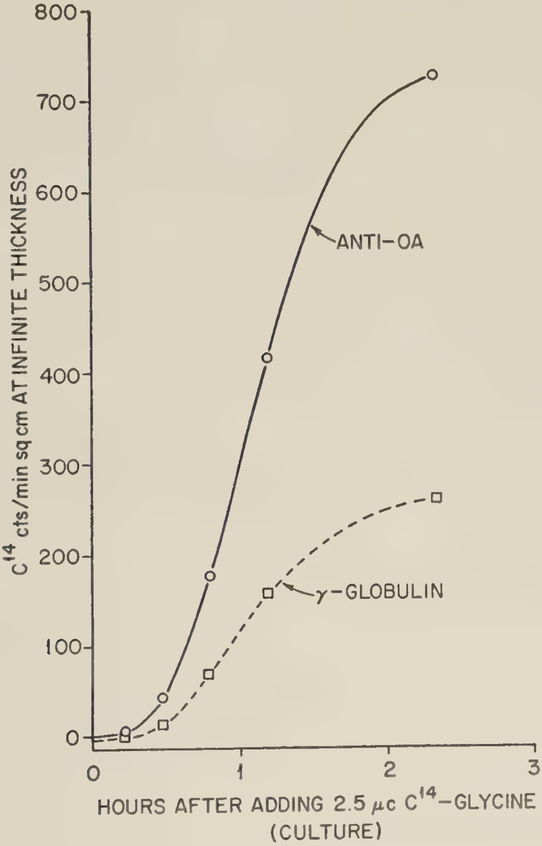

Fig. 8 Comparison of the *in vitro* incorporation of C^{14} into antiovalbumin and the remaining γ-globulin after the addition of 2.5 μc of C^{14}-glycine to preparations of sliced lymph nodes from an immunized donor. (Redrawn from Askonas and White, '56.)

travenously and when there is no long holdup of antibody from the blood, as indicated by the lack of precursors and the short transit time of labeled amino acids during antibody formation, the changes in titer corrected for metabolic decay probably agree well with actual synthesis. Thus Dixon *et al.* ('56) found that the rate of synthesis after peak synthesis, as shown by isotopic measures, fell off rapidly during first and second responses (half life of 1.3 days) and after hyperimmunization. These results are strikingly similar to the postpeak hemolysin decline during the primary response as calculated for the intact rabbit and the spleen separately (fig. 4). They also agree with the slower secondary hemolysin decline during hyperimmunization caused by a relatively greater stimulation of nonsplenic sites (Taliaferro, '55).

OPEN DISCUSSION

OWEN [1]: Is there any reason for believing that the smaller hemolytic antibody may be derived from the larger one?

TALIAFERRO: This possibility has interested Dr. Talmage and me a great deal. We have no evidence, at present, that the smaller molecule is derived from the larger one after appearance in the serum. Mr. Weinrach, have you any ideas on the subject?

WEINRACH [2]: I have nothing definite to suggest that one is derived from the other but it is possible that the larger one can be split into the smaller one *in vitro*.

STELOS [3]: Deutsch and Morton ('57) reported some work that may bear on this question. They worked with the S_{18} component of normal human serum, which is probably similar in sedimentation properties to the large-molecular-weight hemolysins found in rabbit antiserum to sheep red cells. After treatment with mercaptoethanol, the S_{18} component was converted to a smaller molecular entity. It is therefore quite possible that the small-molecular-weight hemolysins could be derived from the larger, and vice versa. But how this conversion could occur physiologically is not very clear to me.

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[1] R. D. Owen, California Institute of Technology.

[2] R. S. Weinrach, University of Chicago.

[3] Peter Stelos, University of Chicago.

PRESSMAN[4]: In connection with the question of S_{18} component versus S_6 component in the hemolytic system, I think it is important to realize that this applies directly to the rabbit hemolysin against sheep red cells. Dr. Johnstone and I made a study of rabbit antibody against human red cells and found that the hemolytic component was almost entirely in the low-molecular-weight fraction.

HAUROWITZ[5]: You said that antibody titer in a serum is a good measure of antibody formation after intravenous injection. In the present situation, we can measure only titers of *circulating* antibodies; but we know that many types of animals, e.g., guinea pigs, do not form circulating antibodies, or form very little. We have proof that some of the antibodies that we find in the circulation in the secondary response have been formed before the second injection and were deposited in the tissue in some form. You may call them dormant antibodies or antigen-antibody complexes. They may be nonspecifically bound to the tissues.

After injection of the second antigen, the bound antibody is released. Therefore, the serum titer of antibody is not a reliable measure of total antibody formed in the organism.

TALIAFERRO: May I say that in the secondary response in the rabbit, the absence of long-lived precursors during the induction period and the transit time of 30 minutes for administered isotopically labeled amino acids to appear in circulating antibody furnish evidence against the idea that antibodies are formed before the injection of antigen. Do you think that some molecules form very slowly or are held up in the tissues?

HAUROWITZ: Yes.

[4] David Pressman, Roswell Park Memorial Institute.

[5] Felix Haurowitz, Indiana University.

DIXON[6]: Dr. Haurowitz, can you be sure in the secondary response that the antibody found labeled with S^{35} was made before the secondary antigenic stimulus and was not the result of recycling of the labeled amino acids from various proteins to antibody made during the secondary response? I think Dr. W. O. Weigle has some evidence that there is an unexpected large amount of recycling of amino acid from endogenous proteins into antibody protein, more than occurs with other serum protein.

HAUROWITZ: We know this from the following experiment, Dr. Dixon: We injected S^{35}-amino acids shortly after the first injection and waited 4 weeks. At that time radioactivity of the nonantibody globulin had gone down and the serum was free of precipitating antibody; it contained traces of coprecipitating antibody. We made the second injection of antigen. Antibody was formed and the specific activity of this antibody was approximately the same as that of the over-all globulin. If the antibody had been formed after the second injection, its specific activity would have been much lower because there was practically no circulating S^{35} after 4 weeks.

DIXON: I don't want to belabor this point, but you say that the antibody that was formed had the same specific activity as the circulating globulin at this time?

HAUROWITZ: Yes.

DIXON: Did you try this in a primary response?

HAUROWITZ: No.

DIXON: We have found large amounts of recycling even in the primary response, where there cannot possibly have been previous synthesis of antibody.

TALIAFERRO: As I understand it, Dr. Dixon, you think that recycling explains the results of Green and Anker?

DIXON: Yes. At least we found it to such a high degree that we have to interpret it this way.

TALIAFERRO: I agree with you but in addition, Dr Talmage and I thought it was labeled coprecipitating and nonantibody protein (complement) that was carried down with the antigen-antibody precipitate.

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[6] F. J. Dixon, University of Pittsburgh School of Medicine.

RAPPORT[7]: I just want to raise a point here concerning the work of Dr. R. R. Porter, in England, who finds that the antibody obtained in the very early part of the response can be separated chromatographically from the antibody that is produced a little later. When I talked to him about 2 years ago, he had also found differences in stability between these two antibodies. Doesn't this suggest that there is a difference in mechanism responsible for producing these two different kinds of antibodies, and perhaps even that different cells are doing it? Do you have any information along this line?

TALIAFERRO: No, none.

HEIDELBERGER[8]: I just wanted to recall that the early results of ours that Dr. Taliaferro referred to on antibody synthesis and half life were obtained with N^{15} and not radioactive markers. These materials and markers had very much less sensitivity than the radioactive markers, and presented both advantages and disadvantages. I think it is all the more gratifying that the use of the very much more sensitive markers gave results along the same lines as the relatively crude methods that were necessary at that time.

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[8] Michael Heidelberger, Rutgers University.

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CHARACTERIZATION OF THE ANTIBODY RESPONSE [1,2]

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FIVE FIGURES

By way of characterizing the antibody response I would like to bring together some of the available information on the disposition of antigen and the synthesis of antibody in a single antigen-host system. The correlation of the data on these two basic processes in a given antibody response may afford a general picture of the antibody response and serve as a frame of reference for some of the more specific topics. The immunologic system I have chosen as a basis for this discussion is the rabbit's response to heterologous serum proteins. The fate of these antigens has been extensively studied by isotopic and immunologic techniques and the antibody response has been investigated from both metabolic and morphologic points of view.

DISPOSITION OF SERUM PROTEIN ANTIGENS

It is generally agreed that after injection most of the antigen material is rapidly catabolized during the ensuing antibody response. There is disagreement, however, on whether small traces of the antigen persist in the recipient for long periods of time. Although this question is difficult to answer directly, correlation of the antigen tracer observations with the rates of antibody synthesis does afford some measure of the amount of effective antigen in the host.

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[1] Those studies carried out in the author's laboratory have been supported by the Atomic Energy Commission Contract AT (30–1)–1205.

[2] Publication No. 122, Department of Pathology, University of Pittsburgh School of Medicine.

Figure 1 shows the familar patterns of elimination of I^{131} trace-labeled rabbit γ-globulin (I*RGG), bovine γ-globulin (I*BGG), and bovine serum albumin (I*BSA) from the circulation of rabbits after intravenous injection. The homologous I*RGG equilibrates between the intravascular and extravascular compartments of the plasma protein pool during the first 2 days (Madden and Whipple, '40) and then is catabolized at a logarithmic rate characteristic of the species. The antigenic proteins, I*BGG and I*BSA, undergo a similar equilibration indicating that they have free access to the same tissues and fluid compartments as do homologous proteins. Coons and associates ('51) showed a transitory appearance of these antigens in the cytoplasm and nuclei of both mesenchymal and epithelial cells, but immunohistologic and I^{131} tracer studies have failed to reveal a concentration or retention of these antigens in any normal cells or tissues. After equilibration, the antigens are catabolized at a logarithmic rate prior to the production of significant amounts of antibody (nonimmune

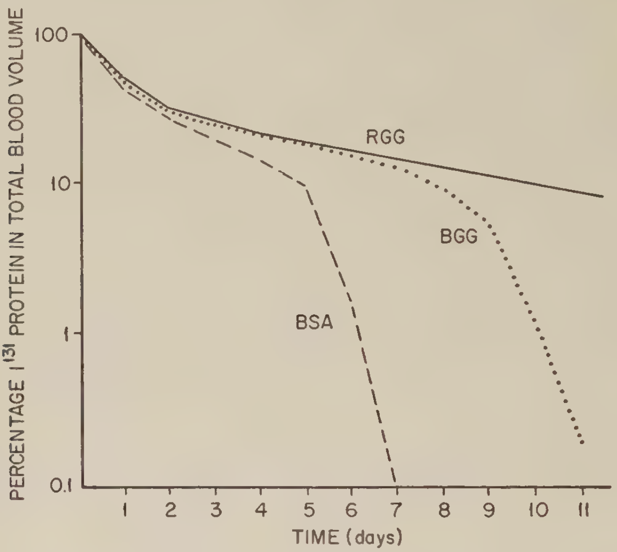

Fig. 1 Disappearance of I^{131} proteins from the blood of rabbits.

catabolism). With the onset of antibody production, the fourth day after injection for I*BGG and the eighth day after for I*BSA, the antigens are rapidly catabolized and disappear from the circulation (immune catabolism) (Talmage *et al.*, '51), after which free antibody appears in the blood.

It has been assumed that these antigens combine with antibody as it is formed and the resulting soluble complexes are then taken out of the circulation and rapidly catabolized (Dixon and Talmage, '51). In general this seems to be true.

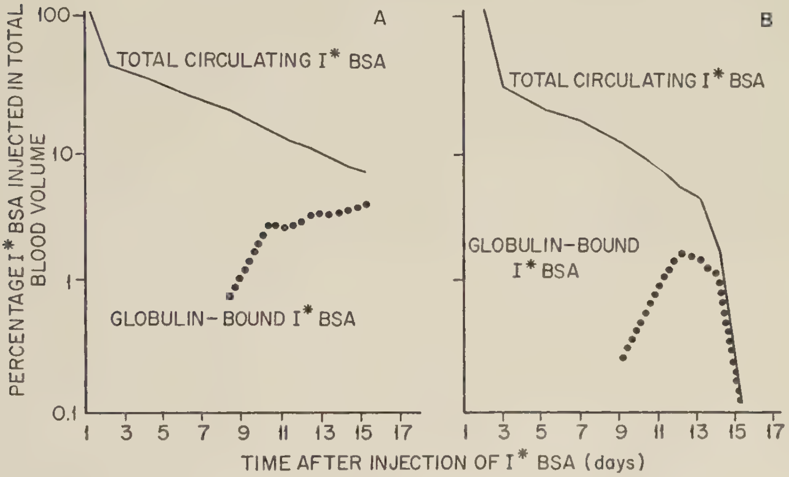

Fig. 2 Circulating antigen bound to the host's globulin or antibody during immune elimination in the rabbit.

Figure 2 A illustrates in a typical rabbit the amount of circulating antigen that may be bound to the host's globulin or antibody during the immune elimination phase. Using the ammonium sulfate precipitation technique of Farr ('56), we precipitated the globulins in the serum of this rabbit at 37°C. and determined the amount of circulating I*BSA bound to the host's globulin (indicated by the dotted line). Just before the onset of immune elimination of antigen, the amount of antigen bound to globulin or antibody increases sharply, and in the final stages of elimination, virtually all of the circulating

antigen is so bound. This straightforward mechanism of antigen elimination, although the rule, has its exceptions (see figure 2B). Here, in spite of the development of considerable amounts of globulin or antibody capable of binding antigen, as shown by the large amount of globulin-bound antigen, there is no demonstrable increase in the rate of elimination of antigen from the circulation. From this it appears that occasional animals either are incapable of removing the usual antigen-antibody complexes from the circulation or, more likely, produce unusual antibodies incapable of forming complexes with the circulating antigen that will be picked up by the host's phagocytic cells.

Active cellular sensitization does not appear to play an essential role in the immune elimination of antigen from the circulation since actively and passively sensitized rabbits with comparable levels of circulating antibody eliminated and degraded antigen at comparable rates. The availability of antibody capable of forming suitable complexes with the circulating antigen seems to be the essential element in immune elimination of antigen from the blood (Dixon and Talmage, '51).

We have not been able to detect circulating antigen or antigen-antibody complexes after the appearance of free antibody. Sternberger *et al.* ('53) have reported the presence of alkali-precipitable material in the serum of rabbits after the appearance of antibody and originally suggested this was antigen-antibody complex. However, using isotope-labeled antigens, Weigle ('57) showed that there is no antigen in these alkali-precipitable materials, and their nature remains unknown. Sternberger ('56) postulated that they might contain antibody and antigen-like materials synthesized by the host.

The fate of these antigens within the tissues is the subject of disagreement. The observations obtained with I^{131}-labeled antigens (Dixon, '54) and immunohistochemical analyses for antigen indicate that these antigens disappear from the tissues about the time free antibody is found in the serum. Briefly, the observations obtained when I*BGG was traced in the tissues of rabbits are shown in figure 3. Figure 3A shows the concentrations of I^{131} in the plasma, liver, and spleen after the primary administration of I*BGG, 30 mg/kg. The levels of I^{131} all drop sharply until the eighth day, at which time antibody appears in the circulation. At this time only a small fraction of 1% of the total I^{131} remains in the rabbit. After the eighth day, the rate of loss of I^{131} is much slower and might suggest the persistence of traces of antigen. As seen in figure 3B, however, these small amounts of I^{131} persisting after the eighth day probably do not represent labeled antigen. Here superimposed on the I^{131} levels found after injection of I*BGG are the levels of I^{131} persisting after the injection of the same amount of I^{131} in the form of iodide (fine lines). Although the amount of I^{131} remaining after injection of iodide was somewhat less than after injection of I*BGG, as might be expected, the rates of loss of the residual I^{131} in both cases were comparable. This would suggest that the traces of I^{131} found subsequent to the eighth day after injection of I*BGG could represent I^{131} liberated by degradation of I*BGG and then incorporated into the host's own constituents.

McMaster and Edwards ('57) found tissue-bound BGG that was still antigenic for rabbits up to 2 weeks after the injection of 100 mg of BGG/kg in rabbits. A BGG-like material that was capable of initiating anaphylactic shock in mice but was not antigenic in rabbits was detectable in the rabbit's tissues for as long as 6 weeks. This latter material was not considered to be the original antigen but perhaps a derivative of it. The difference between the persistence of BGG as detected by the I^{131} label and immunohistologic techniques, 7–9 days, and by the bioassays of McMaster, 14 days, are not greatly different and may be related to the sensitivity of the two techniques employed.

Other investigators used different techniques and found a longer persistence of serum protein antigens in tissues of the rabbits, but these situations may not be entirely comparable to those just discussed. Using proteins coupled by diazo linkage

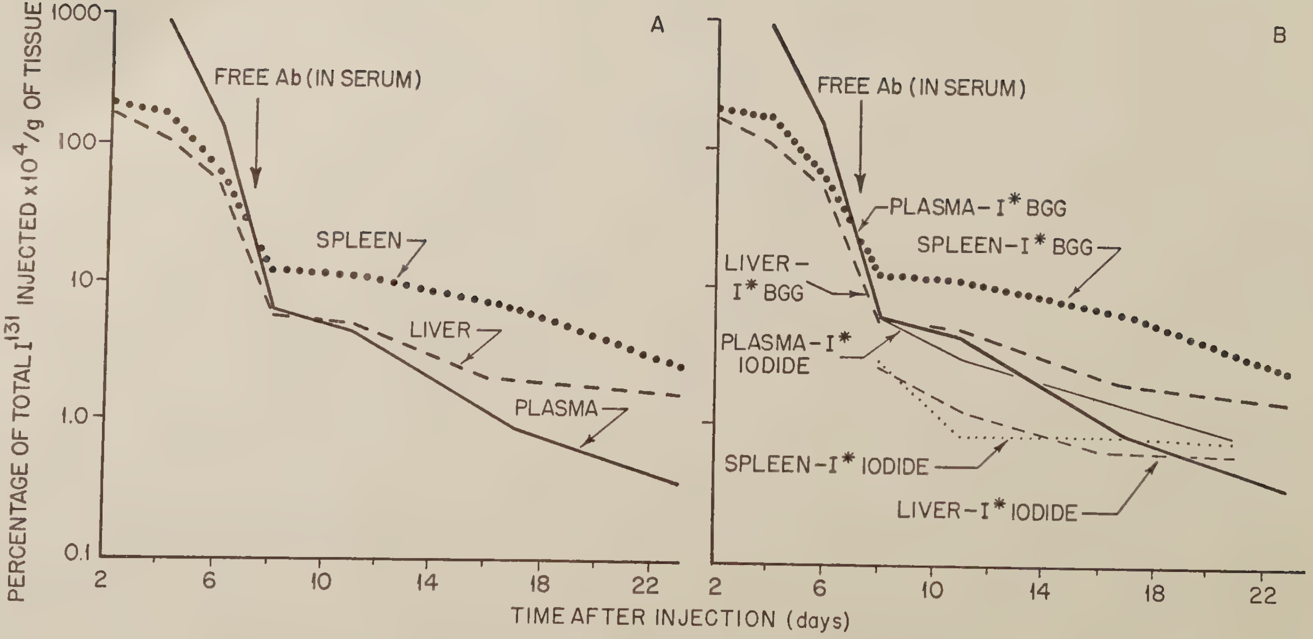

Fig. 3 I^{131} retention in tissues after initial injection of I*BGG.

to a labeled antigenic group, Haurowitz and associates ('55) found antigen or the labeled group to be present in the tissues for considerable periods. D. H. Campbell and J. S. Garvey (personal communication) used similar preparations and found altered forms of the antigen in the tissues for several months. Although the latter materials differed physically and chemically from the original antigen, they did have the immunologic specificity of the antigen. In these cases, it is not certain what effect the azo labeling might have had on the persistence of the antigens.

From these observations, it seems possible that the serum protein antigens in unaltered form do not persist long in the rabbit, but that derivatives of the antigen may be retained in the tissues for considerable periods. However, as we shall see presently, there is little or no correlation between any demonstrated amounts of antigen or derivative of antigen persisting in the tissues and the associated rates of antibody synthesis. If, indeed, some antigen or antigen-like material does persist for a long time after an initial injection, it would appear to be incapable of stimulating antibody production.

Although antigen does not localize or concentrate in normal tissues, it does appear to have a transient localization in the characteristic lesions of serum sickness associated with the injection of large amounts of serum protein antigens. As is well known, the lesions of serum sickness, arteritis, glomerulonephritis, and endocarditis develop during the immune elimination of antigen while antigen-antibody complexes are circulating. By immunohistochemical techniques, antigen and antibody have been shown to localize specifically in these lesions as they are forming. It has been suggested that they localized in the form of antigen-antibody complexes. Since these complexes can initiate both generalized anaphylaxis (Germuth, '57) and marked local inflammatory and necrotizing reactions, their localization in the tissues may play a key role in the formation of the serum sickness lesions. The antigen persists in these lesions for several days and then disappears, so that even here there is no evidence of prolonged retention of antigen. Healing of the lesions accompanies the disappearance of the antigen. It is of interest that the animal referred to earlier, which had the high level of circulating globulin-bound I*BSA without an increased rate of antigen elimination, had a typical serum sickness glomerulitis that we assumed was initiated by the deposition of small amounts of complexes in the glomeruli. Had the serum of this animal been analyzed only for total circulating antigen or antibody, we might have incorrectly concluded that the serum sickness developed in the absence of an immune response; such reports can be found in the literature.

ANTIBODY SYNTHESIS

A good deal of work has been done toward determination of the nature of the biochemical processes involved in antibody synthesis and the actual rates at which these processes operate (W. H. Taliaferro, this symposium). Significant observations on the histologic and cytologic events associated with antibody production have also been made.

The antibody response is characterized by a rapidly increasing rate of antibody synthesis that, in the system we are discussing, reaches a maximum about the time circulating antigen disappears and then promptly declines, suggesting that the major antigenic stimulus is related to circulating antigen. Rates of antibody synthesis throughout primary and secondary responses have been determined by injecting rabbits with S^{35}-labeled amino acids at different times during the response and then analyzing the antibody formed for S^{35} (Dixon *et al.*, '56). Figure 4A illustrates such a set of observations made during the primary response. Each point indicates the S^{35} incorporated into antibody as a result of injection of labeled amino acids on the particular day. The values during the first 3–4 days are inflated by recycling of S^{35}, and should be disregarded. If we accept the observations of Taliaferro and Talmage ('55) of an induction period of 3 days during which little or no antibody synthesis occurs, it is evident that the rate of synthesis rises sharply to peak values between 6

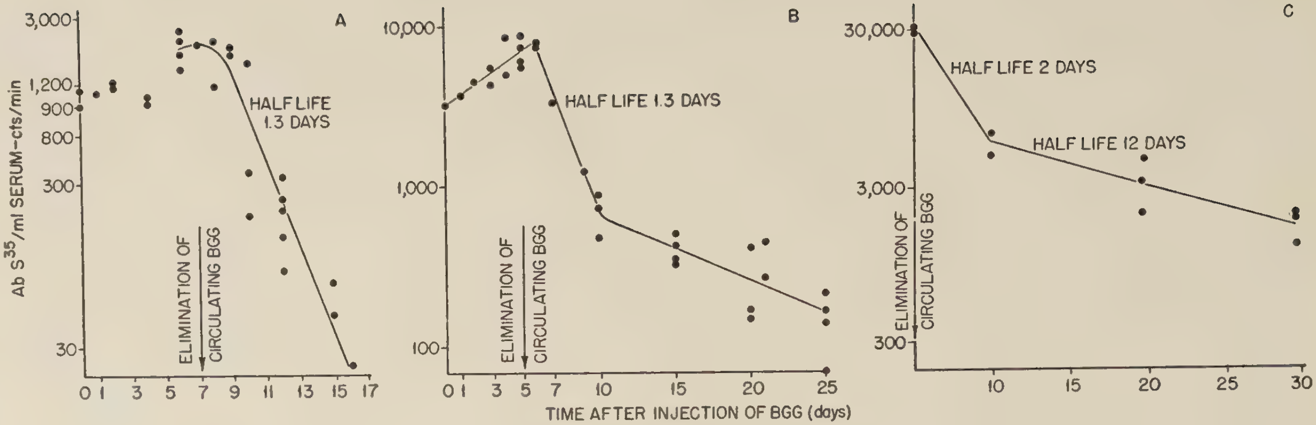

Fig. 4 S^{35} incorporation into antibody expressed as S^{35} counts/minute present in the antibody in 1 ml of serum in each response: primary, A; secondary, B; hyperimmune, C. The S^{35} value is plotted on a log scale according to the day of S^{35} administration. Thus each point indicates the relative rate of antibody synthesis occurring on the day of S^{35} administration. (From Dixon *et al.*, '56.)

and 7 days and then promptly declines. The decline of antibody synthesis that begins with the disappearance of circulaing antigen would seem to argue against any significant persistence of effective antigenic material in the host. Figure 4B illustrates similar observations made during a secondary response to BGG. The same pattern of rise to maximum synthesis and then sharp fall after elimination of antigen is seen here, but instead of the continuing sharp decline in antibody synthesis seen in the primary response, there is a second, slower rate of decline beginning about the tenth day at a level somewhat less than 10% of the maximum rate. Figure 4C based on a hyperimmune response, shows this slow decline of antibody synthesis even more clearly. Here the slow rate of decline begins at a level 20% or more of the maximum rate and there is still considerable antibody synthesis 30 days after intravenous injection of soluble antigen. It is of interest that in all three responses the initial rate of decline of antibody synthesis that starts at the time antigen disappears from the circulation has about the same rate, as though it reflected the loss or decline of something of fundamental importance in all three responses. The level of persisting antibody synthesis in these different responses seems to be related to the extent of sensitization and not to any demonstrable persistence of antigen. By all techniques employed, less antigen was found to persist after secondary stimulation than after primary stimulation, in contrast to a reverse relation for antibody formation.

Two lines of investigation have been particularly productive in defining the numbers and kinds of cells involved in these antibody responses and in determining their antibody-synthesizing capacities. First, as almost everyone will agree, the antibody response is accompanied by the development of elements of the plasma cell series. And, as has been directly demonstrated by Coons and associates (Leduc *et al.*, '55), these developing plasma cells do, in fact, contain and presumably synthesize the antibody. This series of cells usually begins with a large primitive reticular cell that, through mitotic divisions, a reduction in size of nucleus, and an increase in amount of cytoplasm, develops within a few days into a group of typical plasma cells. Along with this morphologic development, the antibody content also increases greatly.

The ability to initiate this morphologic sequence and undertake antibody synthesis may not be the property of only one kind of cell. Although in most situations the primitive reticular cells appear to assume this role, it has been shown that lymph node cells and peritoneal exudates can, after transfer to X-irradiated recipient animals, be driven to or enticed down this familar path (Dixon *et al.,* '57). In these studies, peritoneal exudates (70% macrophages) and lymph node cells (90% lymphocytes) from rabbits previously sensitized to BSA were transferred to the subcutaneous tissues of X-irradiated recipient rabbits and challenged with an injection of BSA. In this situation, the morphologic development and antibody production of a known population of cells could be closely studied. Both the peritoneal cells and the lymph node cells took on the appearance of immature and, finally, mature plasma cells during the course of their antibody responses (Roberts *et al.,* '57). From histologic examination, it appeared that the lymph node lymphocytes and the peritoneal macrophages were giving rise to the plasma cell series. Quantitative determination of the antibody formed by these transferred cells revealed that during the first few days of a secondary response they make an amount of precipitating antibody equal to or greater than their own wet weight. On the basis of the amount of antibody formed and the cell numbers involved, it seems reasonable to us to assume that the lymphocytes of the lymph nodes and the macrophages of the peritoneal exudate are primarily responsible for the antibody formation. Immunochemical analysis of the antibody formed by the macrophages and lymphocyte transfers indicated that both cell types formed antibody of comparable avidity and initial combining ratios. This would suggest that widely differing morphologic entities may have remarkably similar function.

One final point concerning antibody production and associated cellular changes made by Coons and associates (Leduc *et al.,* '55) in the course of their study of the antibody responses to protein antigens in the rabbit's lymph node should be considered. They found, first of all, that prior to antigenic stimulation there were few cells in the lymph nodes of the types known to make antibody. Secondly, in the primary response many cells of the regional lymph node contained antigen shortly after its injection but very few of these antigen-containing cells later synthesized detectable amounts of antibody. However, if a month elapsed after the initial injection of antigen and a second injection was then made, there was a veritable outburst of morphologic and immunologic activity in these same lymphoid cells, resulting in the development of plasma cells and antibody synthesis. Their observations are interpreted as indicating that:

1. There is no cellular evidence of a normal synthetic process that could be modified in its final stages by the presence of antigen to yield antibody, as is postulated by the template theory. There is no evidence that the cell type responsible for antibody synthesis exists in the absence of antigenic stimulation and that the plasma cell series is a specific response to antigenic stimulation. Granting this, the globulin of the plasma cell must then always be an antibody with no inactive or normal prototype.
2. The intracellular presence of antigen *per se* does not result in the formation of antibody directly, but a latent period and a second stimulus appear to be necessary. The cellular events transpiring in this latent period after initial contact with antigen that enable the cell to respond as it does to a second injection is of utmost importance in the understanding of antibody synthesis.

These, then, are some of the more pertinent findings relating to one specific immunologic system. They may afford at least a sketchy outline of the sequence of events—immunological, biochemical, and morphological—that transpire as a result of the intrusion of an antigen upon the biological tranquility of the rabbit.

OPEN DISCUSSION

Talmage[3]: An inspection of Dr. Dixon's curves indicates that the time of maximum antibody synthesis is exactly at the point where antigen disappears, whereas I believe Dr. Taliaferro's curve shows that there is a rise in the rate of antibody synthesis for 24 or 36 hours after that point. Is there a point of difference here?

Dr. Dixon, have you attempted to calculate the rate of antibody synthesis relative to the maximum amount of antigen that could be left in the body? It is impossible to say that at any time there is no antigen left, but it is possible to calculate the maximum amount of antigen left, and knowing the rate of antibody synthesis it is possible to come to some figure of an upper limit of the number of molecules of antigen synthesized per antigen molecule.

Dixon: In answer to the second question, I do not have precise figures of the amount of antibody synthesized in relation to the maximum antigen retained. In the hyperimmune response, when the labeled antigen is eliminated rapidly and antibody synthesis persists for weeks or months, the number of antibody molecules synthesized daily per molecule of labeled antigen remaining would be large indeed. It would amount to several hundred to one thousand antibody molecules synthesized daily per molecule of labeled antigen in the host.

Taliaferro[4]: With regard to Dr. Talmage's first question, we do find a rise in the rate of anti-BSA synthesis for 1–2 days after the beginning of the rise of serum antibody of the secondary response.

Haurowitz[5]: Dr. Dixon made some sweeping statements about the template theory. I would just take up where he left off; namely, with the last few molecules of antigen in the tissue. I think that only these molecules are significant. Deficiencies in our methods make the present experimental work difficult. We cannot find antigen or antibody unless they are free. If they combine with tissue protein, lipid, or nucleic acid, we cannot find them. We have tried in vain to determine serologically radioactive antigen or antibody in tissues. Of course, we find the radioactivity, but this is not a measure of biologically free antigen or antibody.

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[3] D. W. Talmage, University of Chicago.

[4] W. H. Taliaferro, University of Chicago.

[5] Felix Haurowitz, Indiana University.

HEIDELBERGER[6]: It might be appropriate at this point to say a word or two about antibody formation in human beings. The human antibody response to a typical protein antigen, such as diphtheria toxoid, looks exactly like the curves that Dr. Dixon and Dr. Taliaferro have drawn, except that theirs are logarithmic. The ordinate is micrograms of antibody nitrogen per milliliter or whatever the unit is, and the abscissa is time. The initial response to diphtheria toxoid is a curve with a very rapid peak in about 2 weeks and a very rapid decline followed by a more gradual decline.

Jensen calculated that in some of these very high peaks the antibody response might be demonstrated as long as 65 years. He didn't live that long after he made his first measurements, but it is probably a sound calculation that there would be immunity to diphtheria in those cases for as long as 65 years.

If the antigen is a polysaccharide, such as the polysaccharides of the pneumococcus—which are virtually indestructible in the animal body, the picture is very different. There is a rapid rise in perhaps the same length of time, but not quite so high as the diphtheria antitoxin. Then the level of antibody remains at peak values for perhaps 5–8 months, and slowly goes off over a matter of years. Depending on whether one holds to the template theory, one can say that this is perhaps the nearest thing to positive evidence for the template theory, because with antigen for which there is no known destructive enzyme in the animal body, antibody levels remain at their peak for many months and probably fall off only as certain cells die. With protein antigens, however, the greater part of which, at least, are removed by proteolytic enzymes, there is a very rapid decline of antibody levels and then a much slower decline.

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[6] Michael Heidelberger, Rutgers University.

This can all be interpreted as one wishes, I think—on the basis of the Burnet theory or the Breinl-Haurowitz-Mudd-Pauling theory, or any way one wishes.

MAURER[7]: I do not intend to take exception to my teacher's remarks; however, in human beings we find a varied response to a material we know can persist because there is no known system that can degrade it, namely, polyvinylpyrrolidone. Of about a dozen or so people who have produced antibody to this material, some show a response similar to the toxin (protein) system, i.e., a rapid rise, and a rapid fall. Others have a persistence in antibody level for 2 years now. So here is an antigen that is not degraded, as far as we know, yet different persons have different types of responses to this material. This observation does not help us to know whether the template theory can be invoked; it is just another observation (Maurer, '56).

SALK[8]: I did not intend to get into this discussion but since it is obvious that there are differences in the responsiveness of different persons to different antigens, I can cite evidences that suggest the existence of both types of reactions in human subjects, either to infection with polio virus or to vaccination with a noninfectious antigen. I don't know which of the concepts or theories for the persistence of antibody formation is the one that explains all the facts; perhaps both might contribute. Under one set of circumstances, the template theory may be operative; under another, the alternative concept may prevail.

I have a set of observations that I should like to have interpreted by those who are in a better position to interpret them than I.

When in human subjects antigen was injected, a relatively small increment being used between inocula, in doses of 2, 1/2, 1/4, 1/8, and 1/16 ml of antigen, the dose-response effect of primary inoculation was of the order of magnitude seen in figure 5. I think the regression line is such that about a fourfold difference in geometric mean titer of antibody is induced by a tenfold difference in antigen. You can see that it would be difficult to distinguish between two of the doses used, but when put together there is significance to the regression line. When a year later these same subjects were given a uniform dose of antigen, the response was not exactly parallel, but indicated the influence of the prior sensitization of the greater antigenic mass administered initially. At the time of the secondary stimulation, this effect was evident with types 1 and 3, more so with 1 than 3; with 2, the response was fairly uniform. Our interpretation is that we were in a region of antigen excess for type 2, so far as the sigmoid type of dose-response effect is evident, and therefore with the secondary stimulation the type 2 had the maximal effect possible. We happen to have hit, in this very narrow range of dose variation, a portion of the dose-reponse curve that elicited this phenomenon for types 1 and 3.

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[7] P. H. Maurer, University of Pittsburgh School of Medicine.

[8] Jonas E. Salk, University of Pittsburgh School of Medicine.

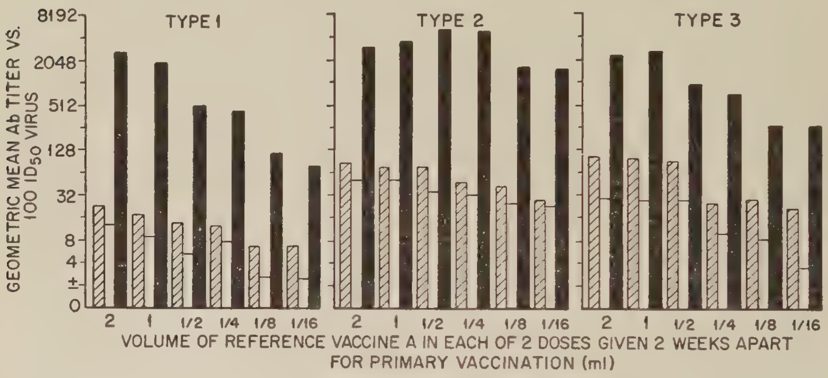

Fig. 5 Influence of primary vaccine potency on booster dose response 1 year later. Shaded columns, geometric mean antibody level 2 weeks after second dose; solid columns, after third dose; open columns, 1 year later. (From Salk, '56.)

The reason this is of some interest is that the secondary response is not an all-or-none phenomenon. This is further evident since there seem to be type-1 antigenic configurations in the type-2 viruses that are naturally prevalent. A person convalescent from a type-2 infection, although he does not posses measurable type-1 antibody for the rest of his life, does have measurable type-2 antibody. Apparently, for life, that individual remains hyperreactive to type 1 in varying degrees. It would appear, then, that the amount of antigenic substance involved in the initial sensitization determines the nature or the degree of secondary response.

I wonder to what extent these observations help to furnish clues to these two different mechanisms.

MITCHISON [9]: Dr. Dixon, is the dose of radiation that you gave to the rabbits sufficient to suppress the graft reaction of the host against the transplanted cells that would normally be accompanied by invasion of the graft by the host?

DIXON: This was presumably the case. We gave 400 r, which has been the usual dose used to suspend the host's immune response in cell transfer studies. However, one does not have to do this. With a nonirradiated recipient, the positive results are comparable, but not quite so many good antibody responses by the transferred cells are obtained, presumably because of occasional host rejection of transferred cells.

HAUROWITZ: Dr. Salk, the antigen retained is, I believe, really decisive for antibody formation and secondary response although we cannot prove it, for two reasons. First of all, antigen deposition may be nonspecific or may take place in cells that do not form antibodies; secondly, there seems to be an enormous difference between the percentage retained of various antigens. Dr. Pappenheimer injected into human beings several micrograms of toxoid and found an enormous response. In rabbits, we inject 100 mg or more. Only a thousandth or a ten-thousandth of it may be deposited and may be significant for antibody formation.

GARVEY [10]: For the past several years, Dr. Campbell and I have been studying the retention and characterization of soluble antigens in the rabbit. The antigens we used were S^{35}-labeled hemocyanin or BSA, prepared by coupling the diazonium salt of S^{35}-labeled sulfanilic acid. These antigens are retained for very long periods of time in the rabbit. In studies made for retention 1 year after the injection of antigen in several-milligram amounts, we have detected levels that were very significant. More than 1 molecule was present per liver cell; so I take exception with Dr. Dixon on the significance of tissue-retained antigen. More than just a few molecules are retained in the rabbit.

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[9] N. A. Mitchison, University of Edinburgh.

[10] Justine S. Garvey, California Institute of Technology.

Referring to one of our papers (Garvey and Campbell, '56), I would like to mention that there is a correlation between retention in liver tissue and the antibody response, at least for 6 weeks after the last of multiple injections of labeled hemocyanin. At such time the circulating antibody had fallen to very low levels (20 μg of Ab N/1 ml of serum). Both the antigen retention and the circulating antibody concentration were leveling out but we would expect that soon the antibody would fall below a detectable level by the techniques that we have available for antibody detection in the circulation.

We have since studied and characterized the retained antigen from livers of immunized rabbits (Garvey and Campbell, '57) and find that in the guinea pig very small amounts of the material will cause sensitization to the original antigen, as tested by the Schultz-Dale technique. Although it persists not as original antigen but as fragments complexed with RNA, the retained antigen was actually more efficient than the original antigen.

PRESSMAN[11]: Along the same line, Dr. Dixon, what seems to be the apparent contradiction that emerges from your data? As I recall, BSA seems to be retained for a somewhat longer period than BGG; yet I think it is common experience that the latter is the better antibody in the rabbit so far as antibody production is concerned.

DIXON: I don't believe there is any evidence that once antibody synthesis begins there is much difference in persistence of the two antigens. The induction period, or whatever happens before antibody synthesis begins, takes longer after an initial injection of BSA than BGG, but once rapid elimination of the antigen begins, both are eliminated from the tissues at about the same rate.

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[11] D. Pressman, Roswell Park Memorial Institute.

As far as BGG being a better antigen than BSA, I don't believe our experience would show much difference between the two, especially with prolonged immunization.

PRESSMAN: I was talking about the primary response, and I think globulin is a better antigen.

WOLFE[12]: I want to differ with the last statement about the antigenicity of γ-globulin and albumin. I do not think there is good evidence for the statement that γ-globulin is a better antigen than albumin. We have injected rabbits and have given chickens primary and secondary injections, but our results show no difference in the antigenicity of the substances, for the amount of antibody nitrogen produced is very similar for both antigens. One must study the curve of circulating antibody, since the peaks do come at different times with different antigens. Our results are based on a single intravenous injection and the animals were injected with comparable amounts of antigen.

FEINBERG[13]: We have been interested in the alkali method of Sternberger and have done several experiments to clarify some of the points Dr. Dixon is concerned about. With the Sternberger technique, antigen did not precipitate spontaneously. It appears, from our data, that the alkali treatment technique does not detect coprecipitating antibody and will not bring down the complex formed in antigen excess.

WAKSMAN[14]: Dr. Dixon finds that cells of the macrophage type and cells that look like lymphocytes can both develop into plasma cells. Many cytologists believe that lymphocytes can mature to become macrophages; and in our work, mononuclear cells obtained from peritoneal exudates (comparable to those used by Dr. Dixon) appear to develop into mature macrophages in tissue culture. When these peritoneal cells are derived from animals with delayed sensitivity and are exposed in tissue culture to antigen, they show an increased rate of maturation to cells of the macrophage type. These findings suggest that perhaps the two cell types in question are not end stages but actually are multipotent cells capable of developing into cells that form circulating antibody, i.e., plasma cells, or of becoming mature macrophages that can react with antigen without releasing antibody. The first type of cellular response would be the characteristic reaction to a secondary antigenic stimulus; the second would be the response to antigen observed in an animal with delayed sensitivity.

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[12] H. R. Wolfe, University of Wisconsin.

[13] Robert Feinberg, Walter Reed Institute of Research.

[14] B. H. Waksman, Harvard University.

WISSLER[15]: Dr. Waksman's comment is very interesting, and I would just like to underline it and perhaps fill in another part of this puzzle. I think a good deal of the difficulty in reasoning here has come about because a good many observations have suggested that the antibody-forming cell is an ''end-stage cell,'' i.e., it develops into a plasma cell and either stays in the tissue or goes on to dissolution. From our observations it seems that the majority of the antibody-forming cells, at least after the first injection of antigen in the rat, develop later into lymphocytes that have exactly the potentiality that Dr. Waksman is describing. They may be carried to other parts of the body where they can become macrophages.

After discussing this problem with Dr. William Bloom, I am beginning to be convinced that some of these macrophages, even though they are phagocytic at one time, can go ahead and form what we call the antibody-forming cell and what he calls lymphoblast.

I believe that we can learn a good deal by studying the cellular aspects of the first response versus the second response in the same species to see if antibody-forming cells are coming from a different source the second time around. It might help with the type of problem that Dr. Salk has outlined and also in a good many other problems.

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[15] R. W. Wissler, University of Chicago.

DIXON: This is a fine suggestion but we cannot get a primary response in our cell transfer system. One would probably have to work with the systems that the Harrises work with or some other similar one in which a bacillary antigen is used. But here the distinct differences between the primary and the secondary responses from an immunological point of view are not apparent. There is question whether a true primary response occurs or whether the usual laboratory exposure of cells from adult animals to bacillary antigens gives a secondary type of response as a result of the animals' previous contact with the same or similar antigens.

PAPPENHEIMER[16]: I should like to amplify some of the remarks made by Dr. Heidelberger. I find it very hard to conceive of antibody production going on in the complete absence of antigen. Dr. Taliaferro's and Dr. Dixon's studies, as well as those of others, have shown that antibody synthesis in response to an injection of a protein antigen usually falls off rapidly soon after the antigen has disappeared. On the other hand, the experiments of Dr. Heidelberger and his group on immunization of man against pneumococcal polysaccharides have shown that antibody production persists for years after a single small dose of antigen. In this case there is good evidence that these polysaccharides are neither metabolized nor excreted.

There are certain conditions under which protein antigens may be presumed to persist for very long periods of time. Freund has shown that rabbits injected with protein antigens incorporated in oil-water emulsion containing killed mycobacteria often maintain a high circulating antibody level for a year or two. Freund's observations suggest that one could always go back and find antigen in the depot.

Some human subjects injected with diphtheria toxoid seem to maintain high levels of circulating antitoxin for many years. Thus one person that Dr. Kuhns and I studied in 1951, whose antitoxin level rose from 1 to 80 units within 14 days after a single booster dose of purified alum-toxoid, still showed

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[16] A. M. Pappenheimer, New York University College of Medicine.

12 units/ml 5 years later. I wonder whether there may be small depots of antigen formed in some persons that escape detection even by tracer techniques and that continue to release traces of antigen over a long period of time.

TALIAFERRO: Antigen depots undoubtedly exist and antibody formation may persist for long periods, but I should like to mention another side of the picture. A refractory period develops in rabbits repeatedly injected with small amounts of antigen in spite of continued antigenic stimulation.

RAPPORT [17]: Just to complete this picture, Dr. Taliaferro, you can administer a complex mixture of antigens in a series of injections; and while you are doing it, antibody against some of the components in the mixture decreases after reaching a certain level.

TALIAFERRO: Just such a condition occurs in our rabbits repeatedly injected with sheep red cells. The Forssman antibody rapidly increases and declines whereas the isophile antibody does not appear until several weeks later.

KOSHLAND [18]: I should like to give an example of diphtheria antitoxin production in the guinea pig where the time sequence is less rushed than in the rabbit. If you measure the concentrations of both antigen and antibody in the popliteal lymph nodes of guinea pigs that have been given a booster dose in the footpad, you get the following picture: There is a rapid disappearance of toxoid in the first 24 hours after injection, followed by a relatively slow elimination that extends for at least 2 months. The antitoxin titer rises sharply to a maximum on the sixth day, falls rapidly until the fourteenth day, and then levels off for the remaining sampling time of 6 weeks.

TALIAFERRO: Is that the antibody curve?

KOSHLAND: Yes. The antibody curve in the lymph node represents the rate of antibody synthesis and is similar in shape to the production curves obtained by Dr. Dixon and Dr. Taliaferro in rabbits. Because the production of anti-

[17] M. Rapport, Sloan-Kettering Institute for Cancer Research.

[18] Marian E. Koshland, Brookhaven National Laboratory.

body is slower in the guinea pig, it is clear from our experiments that there is no direct relation between the amount of antigen present and the burst of antibody synthesized in the first 2 weeks after the booster injection.

However, antigen and antibody do coexist in small amounts for as long as 2 months, and in the later phase their concentrations parallel each other. We think that the slow elimination of the remaining antigen and the low-level production of antibody in this later phase reflect a primary response in new cells not previously exposed to antigen. The time of appearance and the amounts of antibody are very similar to those observed after a primary exposure to toxoid. If this is true, it may be that the antigen-antibody relation is much more direct in the primary sensitization whereas in the booster response some kind of additional mechanism is imposed on this relation.

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PHYSICAL - CHEMICAL STUDIES ON THE NATURE OF ANTIGEN - ANTIBODY REACTIONS [1]

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FIVE FIGURES

The chemical aspects of the reaction between natural protein antigens (Ag) and their antibodies (Ab) may be considered on two levels. The first involves a relatively gross understanding of the reaction, including features such as the nature of Ag-Ab precipitation, and the number of reactive sites, or valences, of Ag and Ab molecules. The second involves finer details of the reaction, such as the exact composition and precise configuration of the reactive sites of Ag and Ab molecules, the energy of Ag-Ab bonds, and the contributions made by various groups to it, and similar matters. It is not surprising that not very much is known as yet about the second level when there is still some controversy over the first. In this paper I shall summarize our studies of the last few years that, by providing direct evidence for the essential correctness of the framework theory of Ag-Ab precipitates, and particularly by demonstrating quantitatively that precipitating Ab is bivalent, have consolidated currently accepted views of the gross features of the reaction. On the second level, these studies have produced reliable estimates of the thermodynamic quantities characterizing protein Ag-Ab reactions; and have provided strong evidence that in each Ag-Ab bond in several different systems there is critically involved a single pair of oppositely charged groups whose chemical nature may be deduced from these and other experiments.

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[1] Contribution No. 1440, Department of Chemistry, Yale University. These investigations were supported by grants from the United States Public Health Service and the Rockefeller Foundation.

Most of these studies have dealt with solutions containing only protein Ag and their rabbit Ab, obtained by dissolving a specific Ag-Ab precipitate in a sufficient excess of the Ag. Our investigations have relied on the resolution and quantitative analysis, by ultracentrifugation and electrophoresis, of the many Ag-Ab complexes present in these solutions.

THE FRAMEWORK THEORY

The ultracentrifuge is an ideal instrument with which to study soluble Ag-Ab complexes, since it separates them primarily according to their masses. From the areas under the peaks of the ultracentrifuge Schlieren patterns one can therefore immediately determine the relative abundance of large and small complexes in a given solution. This was first demonstrated by Heidelberger and Pedersen ('37). A systematic study of these solutions (Singer and Campbell, '52; Oncley *et al.*, '52) at various proportions of total Ag and total Ab provides strong support for the framework theory. In figure 1 are reproduced comparable ultracentrifuge patterns for three different systems, containing bovine serum albumin (BSA), ovalbumin (OA), and β-lactoglobulin (βLG) as protein Ag, and their respective rabbit Ab. Sedimentation proceeds to the left in each diagram.

The bovine serum albumin system. Since BSA has a molecular weight of about 69,000 and Ab 160,000, the free (or uncombined) Ag is the slowest sedimenting species in solution. In heavy Ag excess (fig. 1C) one complex (the **a**-complex) predominates over all others, and its sedimentation constant is consistent with that expected for the $(Ag)_2Ab$ species (Singer and Campbell, '52). (The principal peak in this diagram is caused by free BSA, and no free Ab peak is observed.) In moderate Ag excess (fig. 1B), a number of faster sedimenting complexes appear. Finally, in low Ag excess, close to the zone of precipitation, larger complexes are most prominent (fig. 1A). From equilibrium considerations, it follows that those complexes sedimenting faster than **a** must be poorer in their relative Ag content, and since they are also of larger "molecular" weight, they must contain more than one Ab molecule. Thus the sedimentation constant and other properties of the **b**-complex (fig 1A–C) are consistent with the assignment $(Ag)_3(Ab)_2$, and so on for the larger complexes.

The ovalbumin and β-lactoglobulin systems. The sedimentation diagrams of the OA (fig. 1D–F) and βLG (fig. 1G–I) systems are strikingly similar to the BSA, if the differences in molecular weight of the Ag are taken into account, and confirm that these phenomena are general.

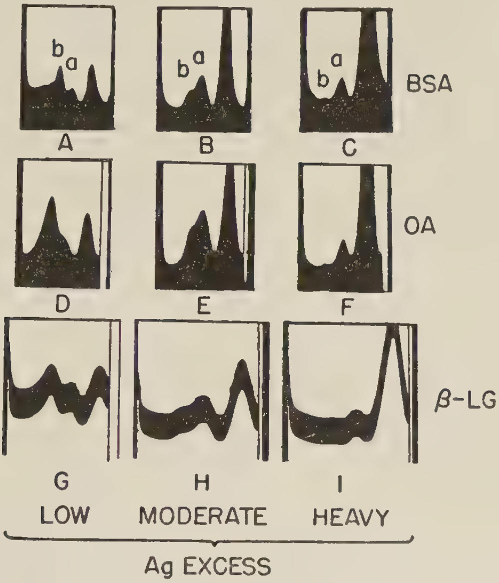

Fig. 1 Ultracentrifuge diagrams of soluble Ag-Ab complexes. Three diagrams are shown for each of three different Ag-Ab systems; along a horizontal row, the degree of Ag excess decreases (the zone of precipitation is approached) from right to left. Within each diagram, sedimentation proceeds to the left. The peak designations a and b refer to specific complexes discussed in the text.

It is clear that these patterns are direct demonstrations of the build-up of a framework of alternating Ag and Ab molecules (Marrack, '38) as the zone of precipitation is approached from the region of Ag excess. As the complexes grow beyond a certain size (depending on the solubility properties of Ag and Ab), they become insoluble and a precipitate forms.

THE VALENCE OF ANTIBODY

Since the **a**-complex predominates in heavy Ag excess, it is the species in which one Ab molecule is bound to as many Ag molecules as its valence permits. The sedimentation studies in the BSA system (Singer and Campbell, '52) indicate that it is the $(Ag)_2Ab$ species, and therefore, that Ab is bivalent.

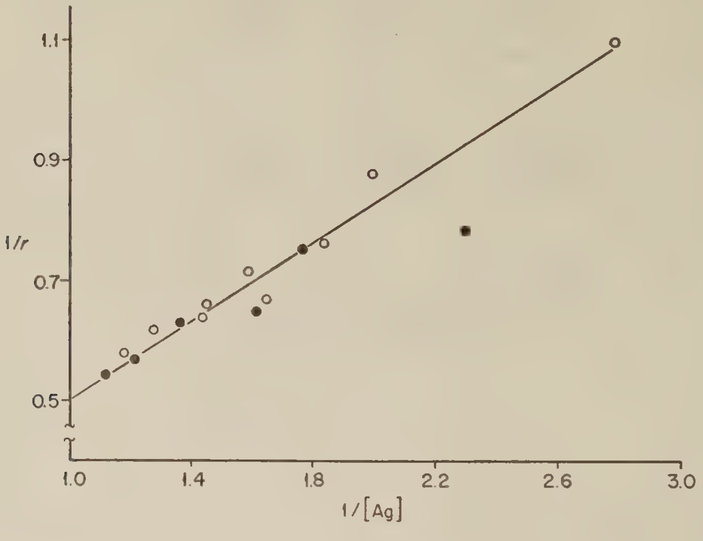

Fig. 2. Binding of Ag by Ab. The ordinates are described in the text. Open circles, BSA system; solid circles, OA system.

The same result may be obtained in an independent way. If we can determine the number, r, of Ag molecules bound per Ab molecule in all the complexes in a given solution, r should approach the value of the Ab valence as the excess of antigen becomes very large. To do this, we accurately determine the equilibrium concentration of free Ag from electrophoresis experiments (Singer and Campbell, '52, '55a; Marrack *et al.*, '51; Plescia *et al.*, '52). Subtracting the free Ag from the total Ag gives the bound Ag, and assuming that all of the Ab is complexed in Ag excess, we can obtain the ratio of Ag to Ab bound in all the complexes of a given solution, which, when multiplied by the appropriate molecular weight factor, gives r. In figure 2, $1/r$ is plotted against $1/[Ag]$, where $[Ag]$ is the concentration of free Ag divided by the concentration of total Ag, for the BSA and OA systems. As $1/[Ag] \rightarrow 1$, $1/r \rightarrow 1/2$, or $r \rightarrow 2$, and each Ab is therefore bivalent. This result is entirely consistent with similar findings, obtained by the entirely different technique of equilibrium dialysis, that antibodies to simple haptens are bivalent (Eisen and Karush, '49; Carsten and Eisen, '55; Karush, '56). On the other hand, it should be emphasized that all these results apply to precipitating antibodies, and do not preclude the existence of other, perhaps univalent, antibodies in special circumstances.

THERMODYNAMIC DATA FOR AG-AB REACTIONS

The forces operating between Ag and Ab molecules in solution can only be inferred from thermodynamic data characterizing their reaction. Rigorous and general methods for obtaining free energy data for protein Ag-Ab reactions have not yet been described. Singer and Campbell ('53, '55a, c) have shown, however, that reliable approximate values may be obtained in the following manner. Goldberg ('52) showed that if all Ag-Ab bonds in a given system are assumed to be intrinsically equivalent regardless of the size of the complex in which they occur, the quantities of all the complexes present in a given solution are simply related to one another. In particular, knowledge of the equilibrium concentration of any one species in a solution of known total Ag and total Ab fixes the concentrations of all others, and hence the equilibrium constants and standard free energy changes. We have already indicated that accurate determination of the free Ag concentration is possible from electrophoresis experiments; these data may then be used to evaluate standard free energy changes. In addition, the effect of temperature on the free Ag concentration, studied in the ultracentrifuge, yields estimates of the enthalpy and entropy changes. In this manner the data of table 1 have been obtained. The assumption of equal intrinsic reactivity of all Ab and Ag sites is undoubtedly only approximately correct. Within the rather large experimental errors encountered in this work, however, no effect of Ab or Ag heterogeneity was observed. The numbers given in table 1 may therefore be considered averaged over any heterogeneity present in the systems.

Several surprising aspects of the thermodynamic data may be noted. The small values of $-\Delta F^0$ of reaction show that in spite of their high specificity, Ag-Ab bonds are relatively weak. Even more striking in this connection are the small enthalpy changes exhibited by these reactions (confirmed by direct calorimetric studies of Steiner and Kitzinger, '56), and the favorable free energy change caused in large part by an entropy increase upon reaction. Generally similar results have been obtained for the reactions of small haptens and antihapten antibodies (Eisen and Karush, '49; Carsten and Eisen, '55; Karush, '56). Furthermore, the remarkable similarity of the thermodynamic data for the unrelated systems of table 1 suggests that it may be generally true that all Ag-Ab bond strengths fall within a relatively narrow range of values. Should this be so, a qualitative explanation might be the following. If the formation of Ab *in situ* requires interaction with an antigen template, then too strong an Ag-Ab bond would

TABLE 1

Thermodynamic data for Ag-Ab systems

SYSTEM	ΔF^{0}[a]	ΔH^{0}	ΔS^{0}
	kcal/mole	*kcal/mole*	*eu*
Bovine serum albumin	-5.5 ± 0.2	0 ± 2	$+20 \pm 8$
Ovalbumin	-5.6 ± 0.2	0 ± 2	$+20 \pm 8$
RBSA-anti-R[b]	-4.8 ± 0.2	0 ± 1	$+18 \pm 4$

[a] For the reaction $Ag + AgAb \rightleftharpoons (Ag)_2Ab$, at 0°C. in veronal buffer, pH 8.6, $\Gamma/2$ 0.1.

[b] Abbreviation for the system containing the antigen benzenearsonic acid-azo-bovine serum albumin and antibodies to benzenearsonic acid. (See Baker *et al.*, '56.)

result in too low a ''turn-over'' number and not enough Ab molecules would be formed and released. On the other hand, if the Ag-Ab bond were too weak, the Ab would be biologically ineffective. Although this hypothesis suggests that the range of $-\Delta F^0$ values might be limited, it does not prescribe the limits, and the need for studies of additional systems is clearly indicated.

THE EFFECT OF *p*H ON AG-AB BONDS

The experiments so far discussed were carried out near neutral *p*H. It is well known, however, that Ag-Ab precipitates dissociate in either acid or alkaline solutions, but no adequate quantitative studies of this effect were made until recently. As will be indicated, under appropriate conditions the effect of *p*H extremes on Ag-Ab equilibria is reversible. Therefore the dissociation is not caused by an irreversible denaturation of Ab. At least three explanations are possible: (1) the Ag and Ab molecules acquire large electric charges of like sign at extremes of *p*H, and the resultant nonspecific repulsion overcomes the specific binding; (2) either, or both, Ag and Ab molecules undergo reversible configurational changes with *p*H that, by diminishing the complementarity of Ag and Ab reactive sites, cause dissociation; or (3) specific groups in the Ag and Ab reactive sites are titrated at the *p*H extremes, and in this state do not permit Ag-Ab bonds to form. Considering the last explanation further, and the following model system, we assume that in each Ag-Ab bond there is a single negatively charged group that is titrated in acid solution, and a single positively charged group that is discharged in alkaline solution. For the moment, let us confine our attention to the acid *p*H region, throughout which the positively charged group retains its charge. If the negatively charged group is characterized by an intrinsic hydrogen ion association constant K_H, and if we neglect the nonspecific repulsion between Ag and Ab molecules, it can be shown that (Singer and Campbell, '55b)

$$\log\left(\frac{1}{K}-\frac{1}{K_0}\right)=\log\frac{K_H}{K_0}-p\mathrm{H} \qquad (1)$$

where K is the apparent equilibrium constant at a given pH for the reaction $Ag + Ab \rightleftharpoons AgAb$, and K_0 is the value of K at neutral pH where both negative and positive groups are fully ionized. A similar relation applies in the alkaline pH region, in which the positively charged group is titrated. It should be noted that if two negative and two positive groups were critically involved in each Ag-Ab bond, the equivalent relation would be quadratic in pH, and would involve a coefficient of two for the term linear in pH.

A quantitative experimental study of the effect of pH on Ag-Ab equilibria may be carried out with the ultracentrifuge. As an example, in figure 3 are reproduced sedimentation patterns

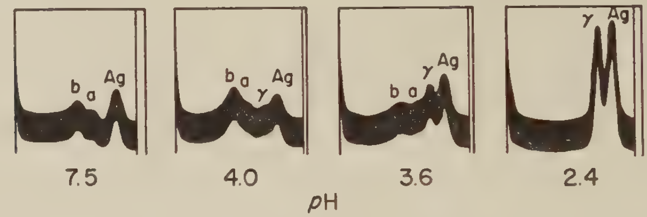

Fig. 3 Effect of pH on ultracentrifuge diagrams in the BSA system. Ionic strength is constant at 0.10. The peak labeled γ represents free Ab. Sedimentation proceeds to the left in each diagram.

of a solution of BSA and its rabbit antibody at various pH values. In the pH range 7.5 to about 4.5, no gross change is observed in the patterns, but at pH less than 4.5, progressively larger amounts of free Ab γ-globulin appear while the complexes diminish. This is clearly caused by a dissociation of the complexes, and the effects are entirely reversible. Enough data may be obtained directly from the relative areas in these patterns to permit evaluation of almost entirely experimental equilibrium constants, K, for the reaction $Ag + Ab \rightleftharpoons AgAb$. In both the rabbit anti-BSA and anti-OA systems (Singer and Campbell, '55b; Singer *et al.*, '55), it is striking that K varies with pH as predicted by equation (1) (fig. 4). For both systems, $K_H \cong 10^5$, a value that is reasonable for a carboxyl group. Although it is not intuitively easy to accept the conclusion that nonspecific repulsive forces are negligible in these systems, there is other evidence supporting this thesis (Singer and Campbell, '55b). We conclude that these data provide strong evidence that a single carboxyl group (and presumably a single positively charged group on the complementary site) is critically present in each Ag-Ab bond in the OA and BSA systems and must be ionized for the bond to form at full strength. Of course this does not imply that structural features other than this ion pair are unimportant. Rather, it suggests that these other features (e.g., hydrogen bonds, dipole interactions, and hydrophobic forces) are directly responsible for only about half, or less than half, of the $-\Delta F^0$ of the reaction.

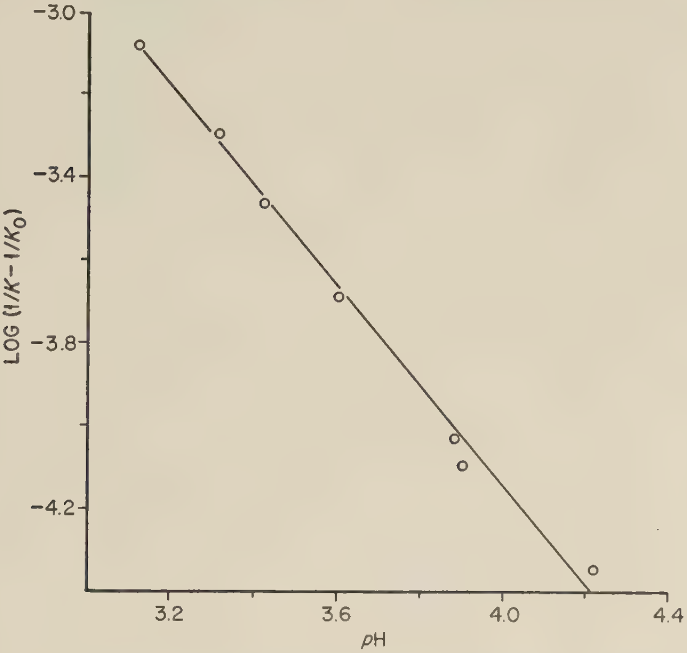

Fig. 4 Effect of pH on Ag-Ab equilibria, for the BSA system, plotted according to equation (1). The slope is −1.2. Reproduced by permission of the Journal of the American Chemical Society.

CHEMICAL MODIFICATION STUDIES

To provide further evidence about the presence of specific charged groups in protein Ag and Ab sites, we have begun a study of the chemical modification of these proteins and its effect on their respective activities. The literature on chemical modification of Ag and Ab is enormous, and cannot be discussed here. Instead, we will confine our attention to a few experiments that we consider most pertinent.

When BSA is acetylated by a procedure (Fraenkel-Conrat *et al.*, '49) that is highly specific for the blocking of free NH_2 groups (α-NH_2, or the ε-NH_2 groups of lysine), and about 75% of its NH_2 groups are reacted, it still precipitates 70% of the Ab precipitable by unmodified BSA (Maurer *et al.*, '57; Singer, '55). Even more to the point, when BSA is treated with o-methyl isourea (Hughes *et al.*, '49), which converts free NH_2 groups to guanidino groups in a highly specific and exhaustive reaction, it retains about 90% of its activity (Maurer *et al.*, '57; Singer, '55). This clearly demonstrates that the NH_2 groups of BSA are not critically involved in Ag-Ab bond formation. It must be pointed out that these results, involving the retention of activity, override older data obtained with less-specific reagents for NH_2 groups with which antigenic activity was destroyed.

When Ab to BSA is acetylated by the procedure of Fraenkel-Conrat *et al.*, it is essentially completely inactivated; that is, it does not precipitate any BSA after 48 hours, standing (Singer, '55). The results of acetylating Ab obtained by Marrack and Orlans ('54) led them to conclude that the Ab was nonspecifically inactivated by acetylation. On the contrary, we consider that acetylation specifically inactivates the Ab, based on the further observation that if the Ab is acetylated in the presence of a large excess of BSA, then despite extensive reaction of its free NH_2 groups, the Ab retains most of its activity (Singer, '55). This is presumably caused by the protection conferred on the critical NH_2 groups in the Ab sites by reaction with the antigen (Pressman and Sternberger, '51). These conclusions are derived from experiments in which a solution containing BSA and its Ab in BSA excess is acetylated and is subsequently examined electrophoretically. The retention of Ab activity is indicated by the persistence of

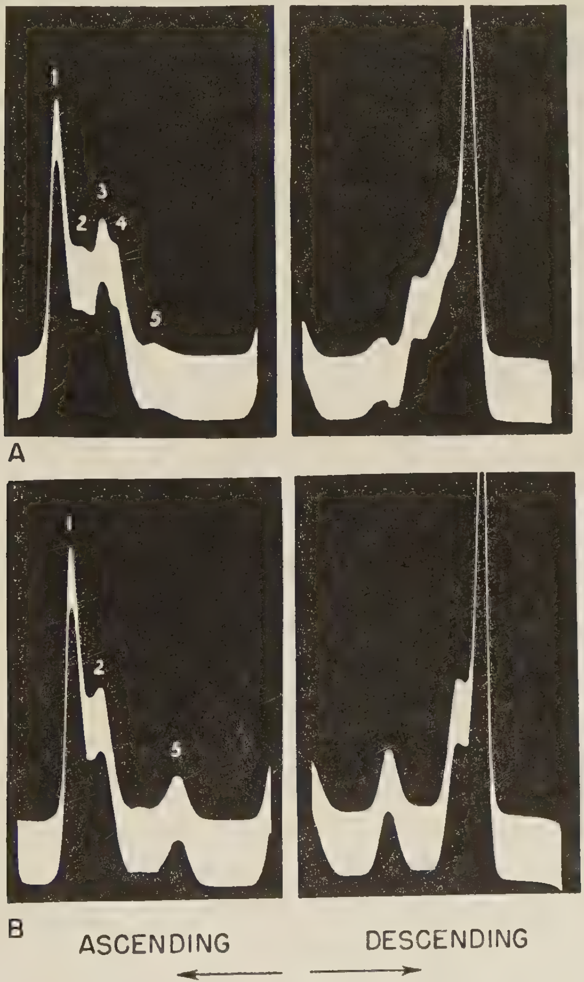

Fig. 5 Electrophoresis patterns demonstrating the protection of anti-BSA Ab by an excess of BSA during acetylation: A, acetylated BSA-anti-BSA complexes; B, a mixture of separately acetylated BSA and rabbit γ-globulin, in veronal buffer, *p*H 8.68, $\Gamma/2$ 0.1. Ascending and descending limb starting positions are indicated by the arrows. Boundaries 1 and 2 are caused by acetylated BSA, boundaries 3 and 4 by acetylated Ag-Ab complexes, and boundary 5 by acetylated γ-globulin. Reproduced by permission of the Proceedings of the National Academy of Sciences.

Ag-Ab complexes, when compared with a solution made by mixing separately acetylated BSA and γ-globulin (see fig. 5).

We have now obtained essentially similar results upon acetylating the antigens: ovalbumin, β-lactoglobulin, bovine γ-globulin, and conalbumin; and their respective antibodies; as well as the system benzenearsonic acid-azo-bovine γ-globulin and antibenzenearsonic acid Ab (RBG: anti-R) (see table 2). Several aspects of these results are worthy of note. In the last-mentioned system, it has long been suspected that there are one or more positively charged groups in each Ab site complementary to the single negative charge (at neutral *p*H) of the benzenearsonic acid hapten. Physical-chemical studies (Epstein and Singer, '58) provide strong quantitative evidence that there is indeed a single NH_2 group in each anti-R Ab site, entirely in accord with the results of the acetylation experiments. This lends further support to a similar inter-

TABLE 2

Effect of acetylation on Ag-Ab reactions

SYSTEM	PERCENTAGE ACTIVITY RETAINED	
	Acetylated Ag[a]	Acetylated Ab[b]
Bovine serum albumin	70	0 [d]
β-Lactoglobulin	100	0
Bovine γ-globulin	76	0
Ovalbumin	78	0 [d]
Conalbumin	66	0 [d]
RBG-anti-R[c]	100	0 [d]

[a] One hundred times the ratio (maximal amount of Ab directed to unmodified Ag, precipitated by acetylated Ag)/(maximal amount of Ab directed to unmodified Ag, precipitated by unmodified Ag).

[b] Amount of acetylated Ab precipitated by unmodified Ag after 24 hours at 0°C. (except in BSA system, 48 hours) above controls.

[c] Benzenearsonic acid-azo-bovine γ-globulin and antibenzenearsonic acid antibodies.

[d] These Ab were shown, by the methods indicated in the text, to be protected during acetylation by excess of homologous Ag. Other Ab was not available in sufficient quantity for similar experiments.

pretation of the acetylation studies in the protein Ag-Ab systems. Furthermore, it is interesting that acetylation of bovine γ-globulin has little effect on its ability to precipitate Ab to the unmodified protein, whereas the acetylation of the rabbit γ-globulin Ab completely inactivates it. This argues against the suggestion that Ab is merely nonspecifically inactivated by acetylation, since acetylation does not affect the properties of γ-globulin, any more than the other proteins examined, as a precipitating antigen.

Our results so far are therefore entirely consistent with the idea that there is a single carboxyl group in each Ag site and a single amino group critically present in each Ab site in the protein Ag-Ab systems examined. Other experiments are in progress or are contemplated to explore this idea further. Quantitative studies of protein Ag-Ab equilibria in the alkaline *p*H range, analogous to those discussed in this paper in the acid *p*H region, are being made. These are more difficult because of the rapid denaturation of Ab in alkaline solution. However, if our conclusions are correct, the results should be analogous to those given in figure 4, with $K_{\Pi} \cong 10^{10}$, characteristic of an ε-NH_2 group of lysine. Furthermore, it follows from our analysis that reagents that specifically block carboxyl groups should inactivate the protein Ag we have so far studied but should permit the retention of Ab activity, provided that they do not cause its nonspecific denaturation. In addition, there are well-known examples of haptens bearing positive charges, and it would be most interesting to determine whether their Ab retain activity upon acetylation. Since one would presume that the charged group in the complementary site would in such cases be negative, the Ab should not be affected by the specific blocking of NH_2 groups.

OPEN DISCUSSION

PRESSMAN[1]: I should like to stress a point about the Goldberg expression. Dr. Singer mentioned that he made the assumption of homogeneity of constants for the various combining sites of Ab and captivity. This is a very great assumption because so many experiments show heterogeneity. Even experiments pointed toward showing homogeneity of Ab always come out with extreme heterogeneity of Ab. On this basis, it is remarkable that he is able to get some constancy in *K*. However, I still think this is rather fortuitous.

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[1] D. Pressman, Roswell Park Memorial Institute.

Another difficulty with the Goldberg expression is that it does not really take into account any cyclic polymer formation. It is all concerned with extended polymer formation.

In connection with the charge interaction in Ag-Ab systems, Dr. Singer has dwelt on the experimental observation that there appears to be a single charge interaction in the site of Ag-Ab interaction, and then he has proceeded to state that through acetylation he found that this is probably a negative charge on the Ag.

SINGER: Acetylation is a reaction that will block the NH_2 groups, which we think are the positively charged groups involved in the Ab sites. We have recently obtained some results indicating that on methylation of the carboxyl groups of BSA and of βLG the antigenicity of these proteins is entirely lost. The methylation reaction is a rather rugged one. One has to demonstrate that this activity is not lost just owing to denaturation of the protein, but I think we have satisfied ourselves about this. So we think that the antigen sites in a number of different proteins contain a negative carboxyl group and the Ab sites contain a positively charged NH_2 group.

SEHON[2]: What about the phenolic groups? Would they not react with the acetic anhydride?

SINGER: Apparently not under these conditions that Fraenkel-Conrat uses, although this is still a possibility.

PRESSMAN: The evidence we have is that in positively charged hapten like the trimethylphenylammonium group there seems to be a negative charge in the Ab site, and that fits in quite well.

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[2] A. Sehon, McGill University.

Another extremely important point that Dr. Singer brought up is the strength of the Ab-Ag bond. This bond seems to be of a mediocre strength, somewhere around 5–10 kcal for $\triangle F$; this is probably quite important in Ab production. If the system for the production of Ab by any kind of template mechanism forms Ab that can bind tightly to Ag, then the system cannot produce much Ab because it would be blocked by a combination with Ag. The Ab cannot be liberated from the system. On the other hand, if the initial mechanism of combination is such as to yield an Ag-Ab complex of too low combining energy, there would be no combination measurable. Finally, if combination constant is of intermediate strength, the Ab can be released and will still combine with Ag but it is of a mediocre combining strength. There is good evidence in connection with Ab against simple substances in such cases. For example, Ab prepared against the *p*-azobenzoate ion will combine with the benzoate ion and also with the substituted benzoate ions. Ab prepared against the 5-azophthalate ion will combine well with the isophthalate ion, but almost not at all with the simple benzoate ion.

Here we need two carboxylates for combination. A single carboxylate charge is not sufficient to hold the Ag-Ab together. From this we gather that antibodies formed against a single carboxylate have a certain energy of combination with the homologous hapten. If the Ab is formed against two carboxylates, an Ab of the same combining strength as above is obtained, but now it is divided between the two charges. Thus a hapten with only a single charge does not combine appreciably with the hapten. This indicates that the attractive force or the combination of each one of the charges to the free energy of combination is less in the phthalate system than in the benzoate system. This is in accord with the generally small range of free energies of combination of Ag with Ab.

Singer: Concerning the very important question of the heterogeneity of Ag-Ab sites — the Goldberg treatment is an approximation, and the only question is whether it is a satisfactory one. Should a theory be developed without the assumption of homogeneous sites, we will apply our data to it. However, the mathematical treatment for a system of heterogeneous Ab is very much more complicated. So of course the temporary justification is to try to use a simple model system. It appears as if the average free energies of combination in a number of different systems is very similar; therefore, it is quite feasible that the distribution of free energy changes in any one system is not very broad. I know that there is some work in the hapten-Ab systems that indicates an appreciable distribution, but that does not necessarily mean that this is true of the protein Ag-Ab systems. In that respect, I wish to emphasize again that the work we have been talking about has dealt primarily with natural protein Ag-Ab interactions, the thermodynamics and characteristics of which are conceivably different from hapten-Ab combinations with which most of the previous work has been done. It is in fact encouraging to see that certain aspects of natural protein Ag-Ab reaction resemble the hapten-Ab systems as closely as they do.

Our free energy changes are to be considered as averaged over any heterogeneity that occurs in the system, and it is likely that our experimental methods are not sensitive enough to pick up the effects of heterogeneity; and anyway these numbers are very crude numbers by standards of a physical chemist. They have quite wide ranges of error and they may incorporate the variations produced by heterogeneity.

PRESSMAN: Actually your K values were very close together. A difference of 2 kcal in ΔF permits a large variation.

SINGER: The other assumption of the Goldberg theory is that cyclic complexes do not form. In our experiments in Ag excess, the aggregates are all fairly small. In a small aggregate, a cyclic structure is very improbable. Whether this assumption is adequate for the precipitation phenomenon is another problem. But applied to soluble aggregates in heavy Ag excess, I don't think it is a critical feature.

Rapport[3]: If the free energy of combination is so small, wouldn't you expect the precipitates to be much more soluble than they are?

Singer: Not necessarily. The free energies we have obtained refer to the formation of a single Ag-Ab bond. With appropriate proportions of an f-valent Ag and bivalent Ab, this free energy makes it very probable that very large Ag-Ab aggregates will form, which should be quite insoluble. In addition, there is the free energy of precipitation that will add to the over-all free energy of the process. In our studies, we have been concerned solely with the homogeneous reaction, all species of molecules being in solution.

Maurer[4]: Both Dr. Singer and I have been working along similar lines with somewhat different techniques. As far as the Ag is concerned, we do agree on the nonimportance of the amino groups (Maurer *et al.*, '57). However, we were struck by the fact that acetylated BSA showed a decrease in cross reaction of about 40% with anti-BSA. From other studies, we attributed the decrease to denaturation of the Ag. This denaturation could not be picked up by techniques other than some I will describe.

I should first like to say that when we used denatured BSA as our Ag and acetylated it, no decrease in cross reaction was observed with Ab against the denatured BSA; also guanidinated BSA showed no decrease in reaction with anti-BSA. In an attempt to get further information on this point, we acetylated rabbit serum albumin (RSA) under so-called mild conditions and immunized rabbits with the acetylated RSA.

Since this material was antigenic in the rabbit, we thought that acetylation was doing more to the protein than just converting NH_2 groups to N-acetyl groups. Sufficient secondary changes seem to have occurred in the molecule to make the material antigenic (Ehrenpreis *et al.*, '57).

We also used isotopic techniques similar to those described by Dr. Dixon. On one of his curves, he described the behavior of homologous I^{131} proteins in a rabbit. If a radioactive (I^{131}) acetylated serum albumin is injected into rabbits, more than 90% of this material is eliminated within the first day, similar to the behavior of denatured proteins. Similarly, acetylated rabbit γ-globulin, which cross-reacts about 60% with sheep γ-globulin, is also eliminiated exceedingly rapidly.

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[3] M. M. Rapport, Sloan-Kettering Institute.

[4] P. H. Maurer, University of Pittsburgh.

I believe we agree on results but differ in the interpretation of the results from chemical modifications of the proteins. It is quite coincidental that the physical-chemical and the chemical modification studies led to the same conclusion. I think the acetylated Ab will not react with the Ag, not necessarily only because the specific NH_2 group may be involved, but because the Ab is also denatured.

SINGER: This is certainly a possible factor that complicates the interpretation of chemical modification studies. However, when BGG is acetylated, it retains 76% of its ability to precipitate with Ab native to the BGG. When Ab is acetylated, it loses 100% of its activity. Assuming that BGG and RGG are similar in their denaturability, one might expect a more complete inactivation of the former. Although the valences of the Ag and Ab are different, this suggests that acetylation is not just causing a nonspecific denaturation of Ab.

More significant is the fact that an excess of an Ag will protect its specific Ab against inactivation by acetylation, according to experiments described in our paper. Thus, although the Ab is heavily acetylated, it retains activity apparently because the specific sites are protected by combination with Ag.

SEHON: You mention that treatment with HCl is reversible. In our laboratory, when we used an antigenically specific ion-exchange resin (similar to the one used by D. H. Campbell), we found that dissociation of Ab with HCl was not quite reversible. In fact, much more precipitation occurred at much higher Ag concentrations, which we attribute to a reduction of the avidity of the Ab. I should like to know whether your systems lead to complete, quantitative reversibility after neutralization of HCl.

Singer: Under the appropriate conditions you can inactivate Ab in acid. Under our conditions, however, we found that keeping Ab at *p*H 3 for the full 24 hours at zero causes only about a 3% loss of Ab precipitability. From the point of view of our experiments, reversibility means that we can recover the ultracentrifuge pattern on neutralizing the solution. This shows that the equilibrium constant has not changed by more than a factor of 2. The equilibrium constant at *p*H 3 differs by a factor of 100 from that at *p*H 7.

Rappaport[5]: We investigated the question of whether denaturation of the Ab or the Ag occurs as a result of acidifying the complexes to *p*H 2.5 or 3.0, using tobacco mosaic virus (TMV) as the Ag. The advantage of a virus in these studies is that the biological property of infectivity can be used as a guide to determine the extent of denaturation. Furthermore, after the Ab has received an acid treatment, and is then neutralized, its ability to inactivate the virus can also be measured. Our data suggest that a two-hour treatment of Ab-TMV complexes at *p*H 2.5 has no effect on either the viability of the virus or the capacity of the Ab to recombine and inactivate the virus. In addition, TMV previously inactivated by antiserum has been reactivated after dissociation and removal of the Ab.

Salk[6]: The combination of the virus with formaldehyde, with 1:4000 formalin at 37° C. and *p*H 7.0, for destruction of infectivity appears to occur rapidly (3–4 days), and it is a reaction apparently of the first order. (About a month after the reaction is set up, there is suddenly a take destruction of Ab-combining capacity.) I wonder whether there is a continuation of the reacton until 100% of the reactive groupings of the virus are involved, thus destroying the biological activity related to Ab-combining capacity, whereas there may be destruction of 75, 80, or 90% of the reactive groupings in the early part of the period, when infectivity only is destroyed.

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[5] I. Rappaport, U. C. L. A.

[6] J. E. Salk, University of Pittsburgh.

SINGER: Although this evidence suggests that perhaps in polio virus NH_2 groups are similarly not essential to its antigenic activity, formaldehyde is not a specific reagent, and it would be hard to say just what the significance of this subsequent drop-off in antigenic activity means.

HEIDELBERGER[7]: I am perfectly willing to go along with Dr. Singer as far as the needs for a carboxyl and an amino group are concerned, but what I wonder is, are they sufficient? I ask because originally Hooker and Boyd calculated that since the bonds responsible for Ag-Ab union are so weak and are probably van der Waals forces or hydrogen bonds, an area of protein surface corresponding to somewhere between 1000 and 3000 in molecular weight would be necessary to hold big molecules together so that they could form aggregates.

If Dr. Singer thinks that a carboxyl and an amino group are sufficient, I would not go along with that, but I would be willing to admit that in the middle of this or somewhere in this area on the Ag or Ab there might be a carboxyl group that is essential, and that in the middle of a similar area on the Ab or Ag there might be an amino group essential for bringing these two together. But the auxiliary forces of larger correspondence in area are necessary to hold two big molecules together so that they can form aggregates.

It is not clear from Dr. Singer's paper, or at least it has not been clear to me, whether he agrees with Hooker and Boyd or whether he thinks that merely the attraction of a single carboxyl and amino would suffice.

SINGER: Our experimental evidence suggests that there is a single negative charge in each BSA or OA antigen-reactive site, and a single positive charge in each Ab site. When the charge in the Ag site is protonated, we observe that the equilibrium constant decreases by at least a factor of 100, corresponding to a change in ΔF^0 of about 2.8 kcal/mole. Since ΔF^0 at pH 7 is about –5.5 kcal/mole, at least half of the free-energy change of the reaction is associated with the interaction of this single pair of charged groups. The remainder arises from interactions of other groups in the Ag and Ab sites. Both sources of ΔF^0 are necessary to produce an effective Ag-Ab bond.

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[7] M. Heidelberger, Rutgers University.

It is also important to note that in many cases, ΔH^0 is small, and ΔS^0 is positive. Where does this entropy increase come from? Two molecules reacting to form one molecule might be expected to show an entropy decrease. One interpretation is that water molecules are present on the reactive sites before the Ag-Ab reaction occurs, held by ion-dipole forces, and are released when the bond forms. Perhaps the steric complementarity of Ag and Ab sites is associated first with bringing the pair of oppositely charged groups into close proximity, and then also with obtaining the maximum release of bound water from the rest of the two reactive sites, resulting in the maximum increase in entropy. Perhaps for this reason, acetate ion, although it has the carboxyl group present in BSA antigen sites, is not a competitive inhibitor of anti-BSA antibody. It does not have the steric configuration to squeeze water out of the Ab site.

HEIDELBERGER: Do you base your statement that ΔH^0 is small on the *K* value calculated according to Goldberg, or does that have nothing to do with the present aspect?

SINGER: No, it is merely a question of how the equilibrium constant changes with temperature, and to estimate this change you have to use some theory, but in a secondary way.

HEIDELBERGER: But as I remember it, your *K* values ranged from 0.5 to 4.4, and to an organic chemist that is anything but constant.

SINGER: On the contrary, to a physical chemist they are not constant. You see, I am in the middle of a dilemma. Physical chemists do not recognize me as a physical chemist and immunologists do not recognize me as an immunologist. But my wife loves me anyway.

HEIDELBERGER: I wondered whether you had made this calculation using both extremes. That is the real point.

SINGER: The temperature coefficient of the equilibrium constant is independent of these variations that you so properly point out exist. We superimpose ultracentrifuge patterns for the same solution taken at two different temperatures and very accurately we can see if the area has changed. In other words, we can determine ΔK in such a case much better than we can determine the absolute value of K. So we can set an upper limit on ΔH^0. Hapten-Ab studies also show that ΔH^0 is often small.

KABAT[8]: I should like to get to this question about the role of the charge: If Dr. Singer is right that this charge contributes about 2-1/2 kcal to the total binding, or 50%, then the question is why do you get a good Ag-Ab interaction with completely uncharged molecules.

SINGER: The mistaken notion is prevalent, I think, that the interaction of two oppositely charged groups in water solution produces a very large energy change. I don't think this is so. If you could substitute interaction of a dipolar nature, such as probably exists with the hydroxyl dipoles of a saccharide hapten, you could conceivably build up enough free energy to give a stable bond. A dipole on a sugar molecule can polarize water, and the entropy release, on formation of a bond with Ab, might then be obtained just as in ion-pair interaction.

It is possible, however, in the protein antigens, that a single ion-pair interaction is often, if not generally, involved in the Ag-Ab bond whereas this is not necessarily the case in other Ag-Ab systems. There are of the order of a hundred carboxyl groups on a BSA molecule, and there are six reactive sites that we think each contain one carboxyl group. How are these particular carboxyl groups selected? It may be that if the interaction between Ag-Ab cannot be too great, because of the requirement that the Ag template release formed Ab, then the carboxyl group that is selected may be one that is relatively isolated from the other charges in the reactive site. If there were other charges in the neighborhood this would cause too strong an interaction.

[8] E. A. Kabat, Columbia University.

Now it may also be that finding an isolated charged group on a reactive site may be relatively rare. In those polypeptides whose amino acid sequences have been worked out, charged groups are often found to cluster. We may speculate that this factor is involved in the process of selection of an antigenic site on a protein. Certainly this would not apply in the case of polysaccharide.

PRESSMAN: In connection with Ab formation against charged groups and uncharged groups, positive charges and negative charges, actually it seems as though you can form Ab against all different kinds of structures. Moreover, it appears as though Ab reacts with the Ag against which it was formed with a certain limited range of ΔF value. If the Ag has a charged group, there will be a large contribution toward the charged group with less contribution toward the uncharged parts. If there is no charge present, there may be a great deal of steric specificity directed toward the hapten because it takes more interaction of the hapten and Ab to give enough force of combination to cause combination.

In this connection, studies of Ab specific to benzoate ion and phthalate ion are pertinent. The haptens are singly and doubly charged, respectively. The antibenzoate Ab seems to fit quite closely around benzoate because if substituents are put into the ortho position, there is quite a decrease of combination. We say that the Ab forms closely around the large part of the benzoate group. With antiphthalate Ab we find that chlorine groups around hapten do not affect the combination much. Even iodine groups do not interfere with the combination. This means that antiphthalate Ab is directed largely against the two charged groups. There is not much specificity or selectivity of this Ab to tell what is sitting on the back of the group. If Ab had formed closely around the rest of the molecule as well as the carboxylates with a lot of specificity, there would have been too much force of combination to permit Ab formation.

This is the basis of the hypothesis that I mentioned previously.

In connection with nature of the site, it is common practice to draw a site that appears to be a definite invagination into an Ab. This frequently cannot be the case because of structural considerations. With certain structures of hapten it is hard to see how Ab of such a close surrounding fit could dissociate from Ag. What is probably happening is that Ab frequently forms against a face of the hapten. We do have evidence in some systems that the Ab forms against one side of a hapten.

SINGER: In connection with the comment that Dr. Pressman made that the release of water with accompanying entropy change would probably involve a corresponding enthalpy change, I don't think that is necessarily true, because when the water is released from the Ag or Ab site to which it is bound by the forces of polarization, it then associates with other water molecules forming hydrogen bonds that are relatively strong. The net effect is the difference between the enthalpy of formation of the water bond to water and the water bond to the haptenic site. That net enthalpy change may be very small but the entropic change will be large.

PRESSMAN: In two substances that can combine with the same site, there will be the same water release, but the extent of combination will be affected by other forces concerned with the ΔH contribution.

SINGER: Not necessarily. If the fit is different, different amounts of water might be released. Dr. Heidelberger has pointed out that in certain pneumococcus polysaccharide (Ab interactions) there is a large temperature effect.

HEIDELBERGER: . . . on the cross reaction. A lot of these that Dr. Pressman has been talking about are cross reactions, too, and I think that a lot of these assumptions may not be valid for cross reactions. The temperature coefficient is enormously different for cross reaction. The entropy changes are very much more exaggerated because of the great, enormous temperature coefficients in some cases.

HAHN[9]: We are all familiar with interactions between proteins that are neither Ab nor Ag. There is a situation where, within the *p*H limits of the isoelectric points, interaction is obtained. Once these *p*H limits are exceeded beyond the isoelectric points of the components, there is dissociation, and also indications of these proteins being in an isolated state and capable of exerting their specific biological activities.

I wonder if your line of reasoning can be applied to the less-specific interaction of proteins within the isoelectric limits as well as to Ab-Ag reactions.

SINGER: This brings up a very interesting difference between nonspecific electrostatic type of interactions and the specific ones. I take it you have reference to something like the situation that obtains when you mix DNA with BSA. At an appropriate *p*H — somewhere below 5.5 and, incidentally, above the isoelectric point of BSA, you begin to get precipitation of DNA and BSA. The DNA is highly negatively charged. The BSA precipitates when it is still negatively charged as well as when it is positively charged. The interaction is caused by a nonspecific electrostatic effect of the charges on the DNA molecule with some charge structure on the BSA molecule. Perhaps the BSA molecule has a local charge density that is positive at *p*H 5.2 or so, even though its net charge is negative. This type of interaction is very sensitive to ionic strength. As the ionic strength is raised, the charges are screened off and interaction diminishes very substantially.

At *p*H 8 with either BSA or OA antigen, both the Ag and Ab molecules bear a net negative charge and, if anything, should repel each other. Yet at this *p*H the interaction is strongest. Furthermore, the ionic strength has very little effect on Ag-Ab equilibria in the same ionic strength range as very considerably affects the nonspecific case. I think this indicates a very different mode of reaction between an Ag and Ab and a nonspecific electrostatic interaction.

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[9] F. E. Hahn, Walter Reed Army Institute of Research.

MAURER: This has to do with modification again. Dr. Singer, what is the extent of the protection against acetylation of the γ-globulin after complexing with Ag?

The other point that has been overlooked here is the paper by Marrack and Orlans, in which a completely opposite conclusion is reached, and I don't know how to explain this. These workers have acetylated Ab, and claim that it still precipitates well with Ag; they conclude that the amino groups on the Ab are not involved at all.

HEIDELBERGER: They did that with only one out of three sera I think.

MAURER: I think about four systems were studied. They studied BSA, OA, a polysaccharide (SIII), and diphtheria toxin.

Getting back to the problem of specific groups, we found that O-acetylation of the hydroxyl groups, either phenolic or aliphatic, of BSA causes a decreased reaction with anti-BSA. When we deacetylate or deesterify under conditions that do not affect the BSA alone, the immunochemical reaction has not been restored. Therefore, something else has happened to the protein besides just blocking a specific group; an irreversible change has occurred.

SINGER: The degree of protection conferred on the Ab by the presence of excess Ag during the acetylation is not yet known. We expect to find it out as follows:

Suppose we acetylate the Ab in the presence of the Ag. If we can now isolate that Ab that is acetylated but that is still an active Ab (we know how to do it, incidentally), I think that we can then do a precipitant titer and find out how much activity is left.

Marrack and Orlans found that acetylated Ab would generally not precipitate directly with Ag, but that it would coprecipitate partially if some unmodified Ab was added. They concluded that this conversion of Ab to an incomplete form by acetylation was a nonspecific effect and was not caused by the blocking of specific NH_2 groups. We do not agree with their conclusion, but more evidence is needed to bear on this point.

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SIZE AND HETEROGENEITY OF THE COMBINING SITES ON AN ANTIBODY MOLECULE[1]

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EIGHT FIGURES

Although the size of the combining site on an antibody molecule, e.g., the characteristic region complementary to the antigenic group, is of fundamental importance, relatively few studies have dealt with this problem and these have been carried out almost exclusively with artifical antigens in which the determinant group was introduced onto the protein. Landsteiner and van der Scheer ('38; see Landsteiner, '45) produced antibodies to an azoprotein in which an arsanilic acid and a succinanilic acid group were attached to the same benzene ring (fig. 1) so that two different groupings that could determine specificity were present. They obtained antibody specific for only the arsanilic acid grouping or the succinanilic acid grouping but not for both. They also prepared antibodies to an azoprotein from *sym*-aminoisophthalylglycylleucine, with identical findings (see also Haurowitz and Schwerin, '43). From these data they hypothesized that the antibody-combining site was not sufficiently large to be complementary to both groups, and Campbell and Bulman ('52) computed that this would fix the upper limit of the antibody-combining site as 700 A ^{2}. Their conclusions are at best only suggestive, since the antibody-combining site might

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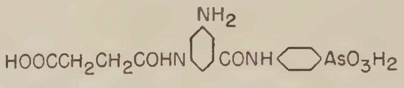
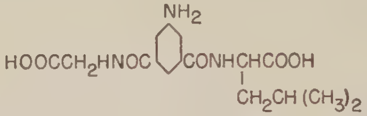
be so restricted in shape that it could not accommodate a haptenic group with two such long side chains. Moreover, studies of Hooker and Boyd ('33) showed that tyrosyl-*p*-phenylarsonate was a better inhibitor of the precipitin reaction between antibodies to protein-*p*-phenylarsonate and azoprotein antigen than *p*-aminophenylarsonate, suggesting that the tyrosine residue by which the azo group was attached to the protein was also part of the antigenic grouping with which the antibody-combining site interacted. Haurowitz and Schwerin ('43) carried out quantitative precipitin and absorption studies with azoproteins containing two types of haptenic groupings and obtained evidence for antibody directed in specificity toward the substituent groups plus some part of the protein antigen. Apart from these studies, Landsteiner and van der Scheer ('32, '34) showed that the terminal amino acid played a major role in the specificity of small peptide chains introduced onto a protein molecule, and Goebel *et al.* ('34) coupled disaccharides such as cellobiose, gentiobiose, lactose, and maltose to proteins via their *p*-aminophenyl glycosides and found that the *p*-aminophenyl glycosides of the terminal nonreducing hexose of appropriate configuration were almost as good inhibitors as the *p*-aminophenyl glycosides of the disaccharides of which they represented the terminal nonreducing residue. Although they drew no inferences from their data as to the size of the combining site of the antibody, the finding



$\mathrm{HOOCCH_2CH_2COHN}\overset{\mathrm{NH_2}}{\bigcirc}\mathrm{CONH}\bigcirc\mathrm{AsO_3H_2}$

(3-AMINO, 5-SUCCINYLAMINOBENZOYL)-*p*-AMINOPHENYLARSENIC ACID (SA)



$\mathrm{HOOCCH_2HNOC}\overset{\mathrm{NH_2}}{\bigcirc}\mathrm{CONH}\underset{\mathrm{CH_2CH\,(CH_3)_2}}{\mathrm{CH}}\mathrm{COOH}$

SYM. AMINOISOPHTHALYL GLYCINE-LEUCINE (GIL)

Fig. 1 Haptenic groups studied by Landsteiner and van der Scheer ('38).

that the *p*-aminophenyl glycosides of the disaccharides were somewhat better inhibitors than those of the monosaccharides would indicate a minimum size for the antibody-combining site of two sugar residues plus the azobenzene ring.

The finding in our laboratory (Kabat and Berg, '52, '53) confirmed by Maurer ('53), that dextran was antigenic in man and gave rise to precipitins, and wheal and erythema skin sensitivity provided a tool par excellence for the further study of this problem. Dextran offered a natural antigen of the simplest possible structure since it consisted of a single sugar, glucose, with α (1 → 6) linkages predominating. Another important advantage in this system was the absence of any charged group on the antigen and hence the elimination of such complications as those caused by binding of albumin. Moreover, at this time the carbohydrate chemists were studying oligosaccharides of the α (1 → 6) series and generously made these materials available to us. The dextrans themselves were also under intensive study at the Northern Regional Utilization Laboratory and many of the ninety-six dextrans classified on the basis of the proportion of 1 → 6, 1 → 4-like, and 1 → 3-like linkages as determined by periodate oxidation (Jeanes *et al.*, '54) were made available to us through the kindness of Dr. Allene Jeanes and Mr. C. E. Rist; Dr. Jeanes also provided a number of the oligosaccharides. Antibodies to dextran were produced in human beings (Kabat and Berg, '52, '53; Maurer, '53), and with these materials available it was demonstrated that the predominant type of immune response yielded an antibody with a specificity directed toward some multiple of the 1 → 6-linked anhydroglucopyranose unit. A single individual produced two types of antidextran, one directed against units in 1 → 6 glucosidic linkage and the other involving units linked other than, or in addition to, the 1 → 6 position (Kabat and Berg, '53; Allen and Kabat, '56).

With so simple a system, the problem of determining the size of the combining site could be reduced to the question of how many glucose residues were necessary to fill the combining site completely. This could readily be determined by a modification of the hapten inhibition technique of Landsteiner ('47) in which the degree of inhibition of precipitation of a given amount of antibody by antigen produced by varying known concentrations of the low-molecular-weight hapten is estimated by the microquantitative precipitin method of Heidelberger and MacPherson ('43; see also Pauling *et al.*, '42; Kabat and Mayer, '48). If the relative inhibiting power of a series of oligosaccharides of appropriate structure and increasing degree of polymerization is measured on a molar basis, the inhibiting power should reach a limit with that oligosaccharide that just satisfied the antibody-combining site; the additional glucose residues of larger oligosaccharides would be outside the antibody-combining site. Smaller oligosaccharides would not satisfy the combining site completely and would be relatively more easily displaced by the larger combining site on the antigen and hence be relatively poorer inhibitors.

The studies shown in figure 2 (Kabat, '54) were carried out with an antidextran of 1 → 6 specificity and clinical[2] or native dextrans from the B512 strain of *Leuconostoc mesenteroides,* which produces a dextran with about 96% 1 → 6 linkages and 4% 1 → 3-like linkages (Jeanes *et al.*, '54). The percentage inhibition produced by varying concentrations (in micromoles) of the different oligosaccharides is plotted. It is evident that the antibody is specific for α (1 → 6)-linked glucoses since oligosaccharides with α (1 → 4) linkages (maltose, maltotriose, maltotetraose, and maltopentaose obtained from Dr. Roy L. Whistler) or with β (1 → 6) units (gentiobiose) did not inhibit or were poorer inhibitors than those with α (1 → 6) linkages. It is also evident that the trisaccharide, isomaltotriose, is a much better inhibitor than the disaccharide, isomaltose, in that only 0.25 μmole of the former was required for 50% inhibition as compared with about 15 μmoles of the latter, when clinical dextran N150N is used. Use of native dextrans produces similar findings. These data established that the combining site on the antibody molecule was complementary at least to a trisaccharide. Since at this time the tetrasaccharide of the proper structure, isomaltotetraose, had not been made, an explicit answer could not be obtained. However, Dr. John H. Pazur, University of Nebraska, kindly provided us with another oligosaccharide, 4-α-isomaltotriosyl-D-glucose. This oligosaccharide is identical with isomaltotriose for the

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[2] Clinical dextrans are prepared from native dextrans by partial hydrolysis with acid and fractional precipitation with methanol or ethanol. They have molecular weights of about 75,000 but the native dextrans have molecular weights in the tens of millions.

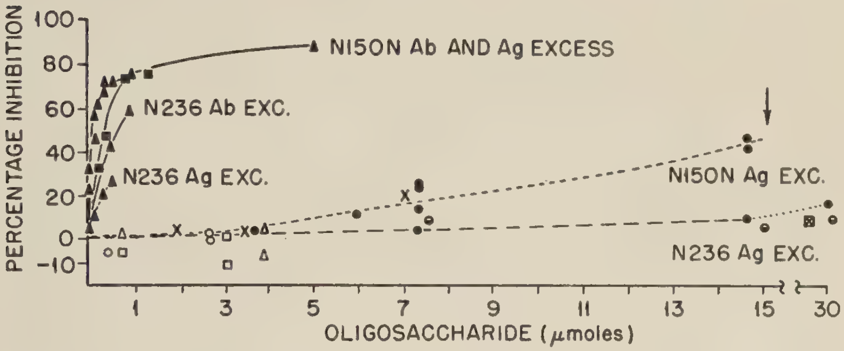

Fig. 2 Inhibition of precipitation of dextran-antidextran $30D_4$ of 1 → 6 specificity by various oligosaccharides; 0.5 ml of antiserum, 4 μg of N150N for antibody excess, 10 μg of N150N for antigen excess, 5 μg of N236 for antibody excess, 25 μg of N236 for antigen excess points; total volume 2.0 ml. △, Maltotriose; □, maltotetraose; ○, maltopentaose; ▲, isomaltotriose; ●, isomaltose; ■, 4-α-isomaltotriosyl-D-glucose; X, panose; ◒, isomaltitol; ⊠, gentiobiose. (From Kabat, '54.)

first three units, but the linkage to the fourth glucose is α (1 → 4) instead of α (1 → 6). It seemed reasonable to infer that if the antibody-combining site of the antidextran were only three units long, the fourth glucose in the α (1 → 4) position should be exterior to the combining site. In this case, the material should be as good an inhibitor, mole for mole, as isomaltotriose itself. If, however, the antibody-combining site were larger than three glucose residues, the α (1 → 4) linkage should prevent the first three residues from fitting as readily into the combining site and thus it should be a poorer inhibitor.

That the latter occurred is evident in figure 2, wherein it may be seen that about three times as much 4-α-isomaltotriosyl-D-glucose as isomaltotriose was required for 50% inhibition. On this basis, we predicted that the combining site was larger than three glucose units and involved at least part of a fourth unit (Kabat, '54). Karush ('56) obtained evidence from studies on inhibition of binding of the haptenic dye with antibodies to D- and L- phenyl (*p*-azobenzoyl amino) acetate (fig.3) and structurally related molecules indicating that the combining region on the antibody "is not much larger if at all than that necessary to make contact with the entire molecule." These results are in good agreement with our findings; it would be of interest, however, to determine the association constant of the haptenic group coupled to tyrosine and compare it with the haptenic dye studied.

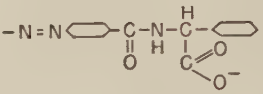

Figure 3

Some 2 years later, J. R. Turvey and W. J. Whelan, University of North Wales, prepared isomaltotetraose, isomaltopentaose, and isomaltohexaose and generously made these materials available to us. The findings with the same antiserum used in figure 2 are shown in figure 4, in which the scale is enlarged. It was naturally gratifying to be able to confirm our prediction, since isomaltotetraose proved to be a better inhibitor than isomaltotriose. The homologous pentasaccharide was better still; and with this serum, inhibition by isomaltohexaose was barely superior to that with isomaltopentaose. It was evident that an upper limit in inhibiting power was rapidly being reached and that this limit corresponded to a hexasaccharide (or possibly a heptasaccharide that has not yet been isolated). This was true whether native or clinical dextrans were used as antigen in the inhibition tests (figs. 2, 4).

In other studies of antidextran sera, it was demonstrated that each person produces a heterogenous population of antidextran molecules, each varying in the extent of its complementary area (Kabat, '56). Were all antidextran-combining sites homogeneous with respect to the extent of their complementary areas, the ratios of inhibiting power of the homologous series of oligosaccharides to one another would be

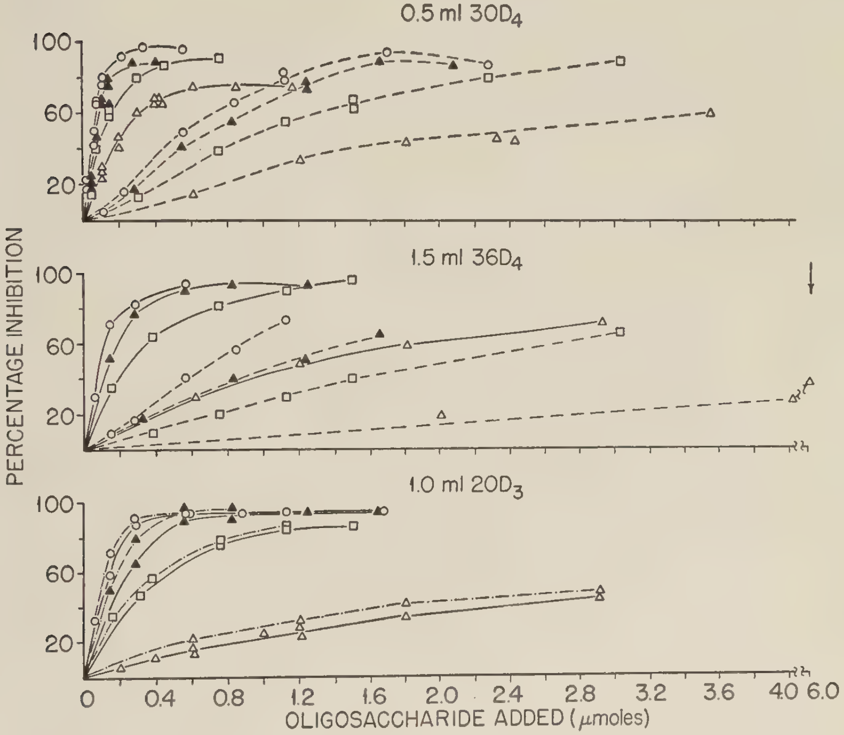

Fig. 4 Inhibition of precipitation of the dextran-antidextran system by the isomaltose oligosaccharides from the tri- to the hexasaccharide. Three antidextran sera are shown: ————, clinical dextran N150N; – – – –, native dextran B512; .–.–.–.–, dextran N150N but a 3 → 4 dilution of antiserum $20D_3$; △, isomaltotriose; □, isomaltotetraose; ▲, isomaltopentaose; ○, isomaltohexaose. (From Kabat, '56.)

constant for sera from different individuals. From figure 4 it is evident that the hexasaccharide is four times as potent as the trisaccharide for antiserum $30D_4$ and about thirty times as effective for antiserum $20D_3$. Antiserum $36D_4$ occupies an intermediate position. If, as suggested by Karush ('56), the antibody-combining site is considered to be a cavity in the regions between the helices into which the antigenic grouping fits, the present data may be explained in either of two ways: (1) that the combining sites actually vary in the size of the cavity, some being large enough to accommodate a hexasaccharide whereas others accommodate only a penta- or a tetrasaccharide, or (2) that the combining sites are all of the same size but the degree of fitting varies with respect to the individual units, the fit being very close for the first three sugars from the nonreducing end, whereas the individual combining sites may differ with respect to the degree of complementariness to the remaining units.

Also of interest in this connection is that the relative proportions of these antibodies with combining sites of different complementariness did not change over a period of 3 years during which no injections of dextran were given; this was observed with subjects 30 and 20 (Kabat, '56).

If a model of isomaltohexaose[3] is constructed, it becomes apparent that the molecule is free to assume a great number of different positions of a random coil, from a very extended structure to one that is compact, as a consequence of the freedom of rotation of each ring around the α $(1 \rightarrow 6)$ linkage (see figs. 5 and 6). The approximate dimensions of the extended hexasaccharide are about 34 by 12 by 7 A. Since to retain its specificity, the antibody-combining site has to be a fairly rigid structure, it is evident that, depending on the relative position of the individual hexose units to one another at the time of antibody formation, an almost infinite variety of antibody-combining sites are possible even though they are all complementary to the hexasaccharide. Whether limitations in structure of the antibody-combining site itself exercise some selective effect and antibodies are formed only to certain

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[3] The author is indebted to Drs. Barbara W. Low, S. A. Barker, A. B. Foster, and M. Stacey for helpful discussions about models.

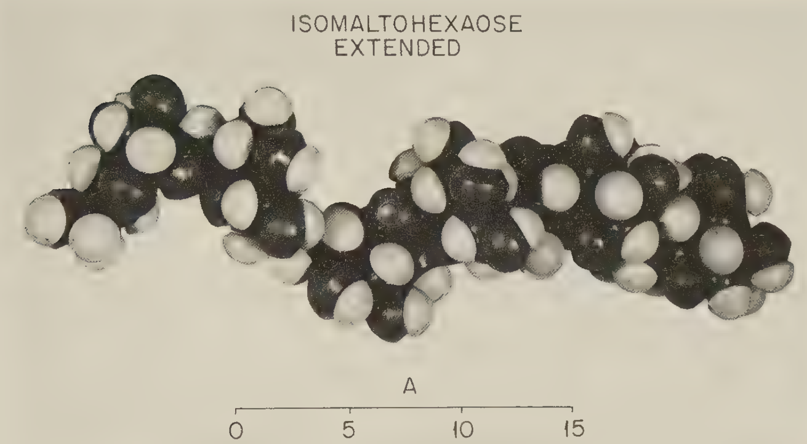

Fig. 5 The extended form of isomaltohexaose.

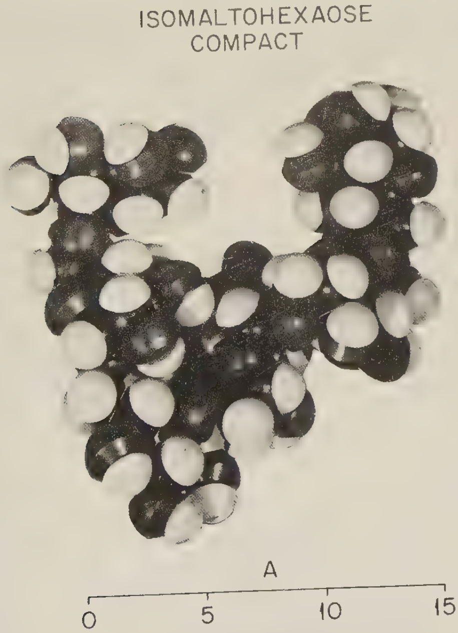

Fig. 6 One of the many more-compact forms of isomaltohexaose.

configurations of the antigenic group cannot yet be determined; despite any such diversities in antibody-combining sites, the antigenic groupings would satisfy them all since they would be free to adjust themselves to the most complementary position within the antibody cavity.

If we accept the hexasaccharide as contributing the maximum-sized group to the antibody-combining site, we may compute the contribution of each glucose residue to binding energy as a Δ (Δ F⁰), comparing each of the oligosaccharides at 50% inhibition; the inhibition data are at equilibrium, since the same degree of inhibition is obtained when the equilibrium is approached in two directions, e.g., addition of inhibitor before antigen and addition of antigen before inhibitor (Kabat, '54). It is evident from table 1 that the first three glucose units from the nonreducing end contribute 90% of the maximum free energy of binding with antiserum $30D_4$ and 75% with antiserum $20D_3$. Additional hexoses contribute relatively less and the sixth hexose contributes only about 2 or 3% to the free energy of binding.

TABLE 1

Decrease in free energy (— ΔF^0) of binding of the oligosaccharides with decreasing degree of polymerization

(From Kabat, '56)

	$\Delta (\Delta F^{\circ})$ (ISOMALTOHEXAOSE[a] — OTHER OLIGOSACCHARIDE)			
OLIGOSACCHARIDE	ANTISERUM $30D_4$	Estimated contribution to total binding	Antiserum $20D_3$ 3→4	Estimated contribution to total binding
	Cal.	%	*Cal.*	%
Isomaltohexaose	..		..	
Isomaltopentaose	160	98	240	97
Isomaltotetraose	330	95	650	91
Isomaltotriose	730	90	1900	75
Isomaltose	3000	60		
Glucose	4600[b]	39		

[a] Assuming ΔF^0 for hexasaccharide = —7500 cal. (Karush, '56).

[b] Estimated from data in Kabat ('54); 50% inhibition estimated to occur at 300 μM.

This system may also be used for attempts to establish the contribution of various individual groupings on the glucose residue to the capacity to inhibit dextran-antidextran precipitation. Figure 7 shows a semilog plot of percentage inhibition against the concentration of oligosaccharide for

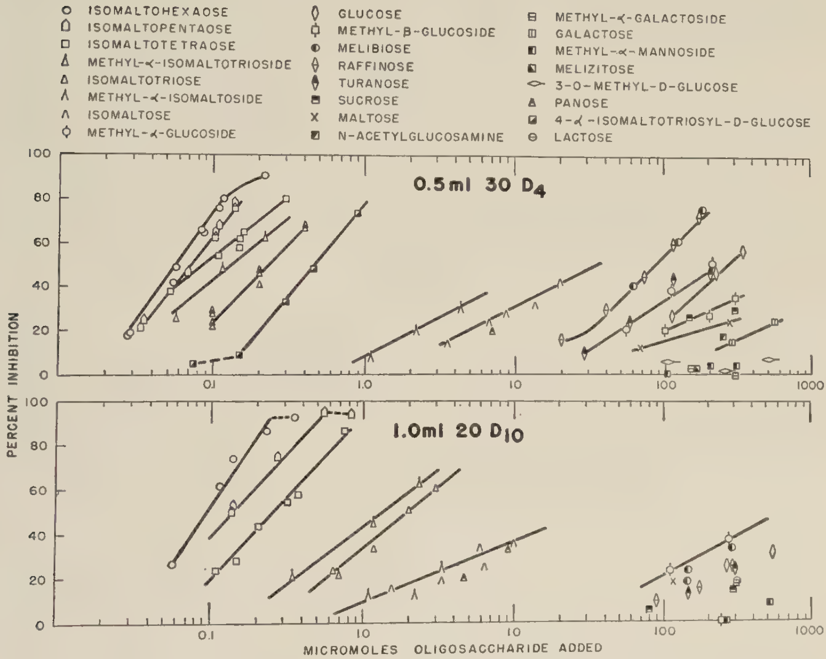

Fig. 7 Inhibition by various mono- and oligosaccharides of precipitation of antidextran by dextran.

antisera $30D_4$ and $20D_{10}$, the latter a sample of serum obtained almost 3 years after $20D_3$. The increase in inhibiting power in going from glucose to isomaltohexaose in the α (1 → 6) series is evident. Also especially satisfying was the finding that methyl-α-D-isomaltoside and methyl-α-D-isomaltotrioside, which had become available through the kindness of Dr. Jeanes (Jones *et al.*, '56) were better inhibitors than isomaltose and isomaltotriose, respectively, and occupied exactly their expected positions in the homologous series. With other sugars, we obtained data about the degree of fitting around the antigen of the antibody-combining sites. Figure 7 shows that with antiserum $30D_4$, melibiose, in which the terminal nonreducing residue is galactose instead of glucose, is a poorer inhibitor than isomaltose; similarly, methyl-α-D-galactoside is a poorer inhibitor than methyl-α-D-glucoside and galactose is a poorer inhibitor than glucose. It is not clear, however, why methyl-α-D-galactoside is poorer than galactose. In models of glucose and galactose, it is evident that galactose is a more compact molecule than glucose. The galactose molecule is thus able to enter into a glucose-combining cavity although it would not be so complementary in structure as glucose, whereas a glucose would not be expected to fit into a galactose-combining site. In studies on the human blood group B anti-B system (Kabat, '57), this latter has actually been observed, e.g., methyl-α-D-glucoside is an extremely poor inhibitor relative to methyl-α-D-galactoside. The importance of carbon 2 is also shown by the very much lower inhibiting power of methyl-α-D-mannoside as compared to methyl-α-D-glucoside. Substitution of a methoxyl for the hydroxyl group on carbon 3 to give 3-O-methyl-D-glucose also produces a very poorly fitting molecule. Differences in architecture of the antibody-combining site are also evident from a comparison of the findings with the various sugars for the two sera. Thus, in antiserum $20D_{10}$, methyl-α-D-isomaltoside is not appreciably better than isomaltose, and melibiose is no better than methyl-α-D-glucoside. The latter antiserum is the one with more antibody-combining sites with areas complementary to the hexasaccharide, and it is not surprising that changes in a single unit should produce much less-marked effects.

Another question that arises is whether the data on the size of the antibody-combining site to the α (1 → 6)-linked glucose chain hold for other antigen-antibody systems. Materials and structural information are not available for proteins, and Maurer ('56, '57) has not been able to secure the necessary low-molecular-weight polymers of N-vinylpyrrolidone to study in the PVP-anti-PVP system. We have, however, studied the serum of a person who produced antidextran with specificity involving 1 → 4-linked units and obtained evidence that the antibody-combining site was complementary at least to a tetrasaccharide (Kabat, '54). These data were based on a correction for partial splitting of the α (1 → 4)-linked oligosaccharides by serum amylase under the conditions of the assay. Figure 8 shows the inhibition findings with this antidextran and a dextran NRRL B1299 S-3 showing 50% 1 → 6- and 50% 1 → 4-like linkages by periodate oxidation. The 1 → 4-like linkages have been shown to consist of 12% 1 → 4 and 38% 1 → 2 linkages (Scott *et al.*, '57) from optical rotation measurements on their cuprammonium complexes. Infrared studies also indicate the presence of some 1 → 3-like linkages (Jeanes *et al.*, '54). It is evident that the maltose series of oligosaccharides inhibit most strongly whereas the slight degree of inhibition with 1 → 6-linked oligosaccharides is caused by a small amount of antibody of 1 → 6 specificity. Although maltotriose, maltotetraose, and maltopentaose appear of equal potency, the amylase in the serum split 35% of the

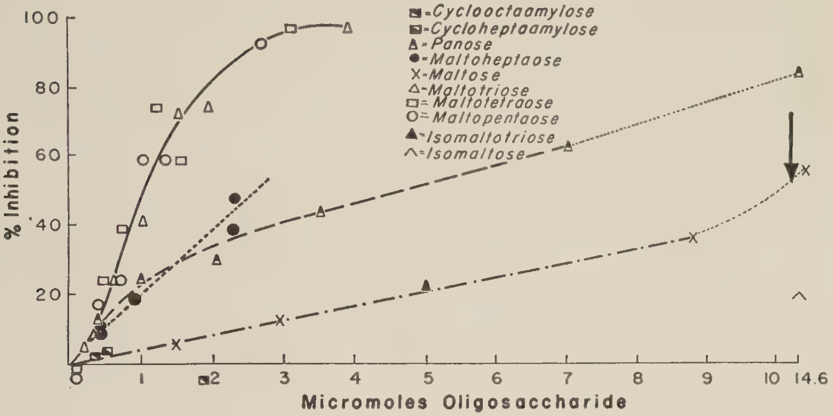

Fig. 8 Inhibition of precipitation of dextran-antidextran $9D_4$ of 1 → 4 specificity by various oligosaccharides: 1.0 ml of antiserum, 100 μg of dextran B1299 S-3 containing 50% each of 1 → 6 and 1 → 4-like linkages; total volume 2.0 ml. (E. A. Kabat, Journal of the American Chemical Society, *76:* 3709, 1954).

maltotetraose and 60% of the maltopentaose under the conditions of the experiment. Had there been no amylase present, the maltotetraose would probably have been about one-third better than maltotriose on a molar basis, suggesting a combining site of more than three 1 → 4 α-linked glucoses. Of especial interest is the finding that maltoheptaose is a poorer inhibitor, on a molar basis, than the corresponding triose, tetraose, or pentaoses. If maltoheptaose were completely split by amylase, one molecule of maltotriose would be formed and it should have been as active as the triose. The finding that is is a poorer inhibitor indicates that it is not completely split and suggests that its lower inhibiting potency may be caused by steric hindrance; it is the shortest oligosaccharide of the maltose series that has the capacity to assume a helical structure. This would also suggest that the antibody cavity is complementary to a random coil.

In the levan human-antilevan system (Allen and Kabat, '57), Peter Allen, Shlomo Hestrin, and I (unpublished data) found that a mixture of oligolevans with 5–7 units is better in inhibiting power than oligolevans with 2–3 fructose units.

In studying the cross reaction of dextrans with type II antipneumococcal serum, Goodman (Goodman and Kabat, '57) found that isomaltopentaose and isomaltohexaose are somewhat better inhibitors than isomaltotriose and methyl-α-D-isomaltotrioside, again indicating that units as large as penta- or hexasaccharides can enter into a type II antibody-combining site.

The conception of the antibody-combining site as a cavity complementary to an open chain of up to about six hexose units imposes distinct limitations on the kinds of structures to which antibody-combining sites may be formed and with which they can show cross reactivity. For example, it becomes very difficult to see how 1, 4, 6 branch points in glycogen could enter into the antibody-combining site of type II antipneumococcal serum and be responsible for the cross reactivity if all the 1, 4, 6 branch points serve to connect long chains of 1 → 4-linked glucose units. Such cross reactivity would readily be explained if the cross reaction were caused by a single glucose unit linek α (1 → 6) at the 1, 4, 6 branch point or to occasional short chains of two or three α (1 → 6)-linked glucoses; the latter possibility being suggested by the isolation of isomaltotriose from glycogen by Wolfrom and Thompson ('56). This interpretation is in accord with the findings of Heidelberger *et al.* ('56, '57) that limit dextrins are more effective than glycogens in cross reacting with type II antiserum as well as with those of Hehre ('56), suggesting that the cross reactivity of dextran with type II rabbit antibody is dependent on α (1 → 6)-linked glucose units. Moreover, Goodman and Kabat (unpublished) found that the various oligosaccharides used in the dextran-type II cross reaction inhibit the glycogen type II cross reaction in about the same sequence, but that very much lower concentrations are required with glycogen than with dextran. This latter finding would suggest that the cross-reactive groups in glycogen are shorter or fewer in number, or both, than those in dextran in which the cross reactive unit is probably at least a terminal isomaltose linked α to the third glucose unit, a conclusion supported by the necessity of using much larger amounts of glycogen than of dextran to precipitate the cross-reacting antibody (Heidelberger and Aisenberg, '53; Heidelberger *et al.*, '54).

The inhibition technique may also be used in reverse to elucidate the structural units involved in other antigen-antibody systems by ascertaining which structural units optimally satisfy the antibody-combining site in these systems. As examples, our quantitative inhibition studies on the A-anti-A, B-anti-B and partially degraded blood group systems (Kabat and Leskowitz, '55; Kabat, '57; Allen and Kabat, '57) as well as the qualitative inhibition studies of Morgan and coworkers (Côté and Morgan, '56) have yielded important clues about the structure of the oligosaccharide side chains determining blood group specificity and those involved in cross reactivity with the plant hemagglutinins (Morgan and Watkins, '53) and with type XIV antipneumococcal serum (Watkins and Morgan, '56; Allen and Kabat, '57, cited by Kabat, '57). Similar extremely interesting studies have thrown light on the nature of the groupings in the group specific carbohydrate of group-A streptococci that determine the specificity of typical and variant strains (McCarty, '56; McCarty and Lancefield, '55). The quantitative precipitin studies on cross reactions of glycogens, polyglucoses, plant gums and other polysaccharides with various types of antipneumococcal sera (Heidelberger, '55, '56; Heidelberger, Adams, and Dische, '56) provide an independent approach to finding out about the groupings responsible for such cross reactions and the final interpretations must satisfactorily account for data obtained by both types of immunochemical investigations. It is obvious that an increased knowledge of the dimensions, architecture, and heterogeneity of antibody-combining sites will play an important role in our understanding of the relations between immunological specificity and chemical structure.

OPEN DISCUSSION

Singer[4]: I should like to raise a question in connection with hapten inhibition studies in general, which may have some bearing on the quantitative interpretation of Dr. Kabat's results.

Consider the reaction of two different haptens, X and Y, with the same antibody, Ab:

$$\begin{array}{ccccc} X & + & \mathrm{Ab} & \rightleftharpoons & X\mathrm{Ab} \\ (\overline{\mathrm{F}}_X) & & (\overline{\mathrm{F}}_{\mathrm{Ab}}) & & (\overline{\mathrm{F}}_{X\mathrm{Ab}}) \end{array} \qquad (1)$$

$$\begin{array}{ccccc} Y & + & \mathrm{Ab} & \rightleftharpoons & Y\mathrm{Ab} \\ (\overline{\mathrm{F}}_Y) & & (\overline{\mathrm{F}}_{\mathrm{Ab}}) & & (\overline{\mathrm{F}}_{Y\mathrm{Ab}}) \end{array} \qquad (2)$$

where $\overline{\mathrm{F}}_X$ represents the partial molal free energy of component X. In discussing the relative "binding affinity of X and Y for Ab, we would like to compare $\overline{\mathrm{F}}_{X\mathrm{Ab}}$ and $\overline{\mathrm{F}}_{Y\mathrm{Ab}}$; however, what we actually measure corresponds to Δ F (reaction 1) $= \overline{\mathrm{F}}_{X\mathrm{Ab}} - \overline{\mathrm{F}}_{\mathrm{Ab}} - \overline{\mathrm{F}}_X$ and Δ F (reaction 2) $= \overline{\mathrm{F}}_{Y\mathrm{Ab}} - \overline{\mathrm{F}}_{\mathrm{Ab}} - \overline{\mathrm{F}}_Y$. Thus to compare the "binding affinity" of the two haptens, we must also know independently the numbers $\overline{\mathrm{F}}_X$ and $\overline{\mathrm{F}}_Y$.

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[4] S. J. Singer, Yale University.

Speaking very crudely, the point is this. If, let us say, one of the two haptens is less water soluble than the other, and we think of forming a hapten-Ab bond as a way of precipitating it out of solution, then the equilibrium constant for the formation of the hapten-Ab bond is favored for that hapten, even though the bond strength might be intrinsically the same for both hapten-Ab combinations. Thermodynamically, this means that the less-soluble hapten has a higher partial molal free energy in solution. In a homologous series of haptens, we would expect the solubility to decrease with increasing molecular weight.

Kabat: I think in this case it does not.

Singer: In any event, unless the partial molal free energies were identical for the two haptens, their difference would contribute to the difference in the observed hapten binding.

A particular case in point occurs in some work of Karush ('56), who finds that the removal of a benzoylamine group from a hapten, leaving a phenylacetate structure, causes a marked reduction in the binding to the same Ab made to a related molecule. A substantial part of this reduction may be attributable to the larger value of the partial molal free energy of benzoylaminophenylacetate than of phenylacetate in solution, and not to the hapten-Ab bonds as such being different.

Kabat: I discussed this point at considerable length in my paper in the December Journal of Immunology (Kabat, '56). Actually a plot of the log of a partition coefficient (α'), e.g., log ($R_f/1 - R_f$), against the degree of polymerization for a series of oligosaccharides would show that the increment, which would be again a $\Delta\ (\Delta F^0)$, would have a negative slope showing that increasing chain length favors aqueous solubility. This is in the opposite direction from what you would predict on the basis of this analysis.

The studies of French and Wild ('53) and Weissmann *et al.* ('54) in making such measurements have shown that this change is linear per unit as chain length of the oligosaccharide chain is increased and the sugars become increasingly water soluble. In our studies, it very definitely can be seen that we are approaching a limit as the chain length increases, and the effect is in the other direction.

Moreover, in regard to the so-called solubility effect— in comparing the proper tetrasaccharide (isomaltotetraose) and 4-α-isomaltotriosyl-D-glucose, the solubility difference just from this slight linkage difference is infinitesimal and yet the effect on the inhibition of this change from the right structure to the wrong one is tremendous compared with what you would have reason to expect on the basis of solubility.

SINGER: This is precisely the point. In certain cases, the effect I have been talking about may be negligible; in other cases it may be substantial, but it should not be ignored.

PRESSMAN [5]: The problem that Dr. Kabat has proposed to study here, the exact size of the whole Ab-combining site, is a very difficult one to tackle. Actually, one can get information about what is required for a combination of hapten and Ab from the study of various haptens, but it is difficult to obtain a limiting upper value for the size of the combining site. For instance, a haptenic substance may more than fill the actual Ab site and thus extend beyond. The part that extends beyond may interact with the rest of the Ab molecule to give additional attractive force owing perhaps to van der Waals interaction or repulsion caused by steric interference. Dr. Kabat has taken any increase in combining force with increased size of hapten to be attributable to increased interaction with the Ab-specific region, and this may not be strictly true.

I should like to comment also on the heterogeneity of the Ab system Dr. Kabat mentioned. He has plotted the percentage inhibition of the precipitation by small substances against the log of the concentration and obtained a series of straight lines. Although many of these lines are roughly parallel, some have a slope that is different from the others. This difference in slope arises directly from the heterogeneity of the Ab in the system. We have been obtaining information on heterogeneity by such plots since 1944. If two haptens are studied in their combination with the Ab in a particular serum and their combining constants for each Ab molecule are in constant ratio, the curve obtained will be parallel. However, if the one substance being investigated combines relatively more strongly than the other substance at one site than at another, one obtains a line of a different slope that is caused by heterogeneity, as in Dr. Kabat's data.

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[5] David Pressman, Roswell Park Memorial Institute.

There is much information for heterogeneity of Ab from previous work, and I think there is a very nice example in the first slide that Dr. Kabat showed. If I may have it, I will use your slide (fig. 1). This is from Dr. Landsteiner's work, and the experiment that Dr. Kabat mentioned does show heterogeneity very well. I would like to correct Dr. Kabat's statement about Dr. Landsteiner's study because I have used this same example, but I have always used it differently — I think the way Landsteiner used it! Dr. Kabat, as I remember, you said that the glycyl-leucyl substance was coupled to a protein and, when injected, the Ab that were formed would react with either the leucyl or the glycyl end but no Ab against the whole.

This is incorrectly stated but it is true that the system shows heterogeneity, since Landsteiner and van der Scheer found that the Ab formed against this could be fractionated; that is, they could absorb out Ab that would react with the leucyl part. They could then absorb out Ab that would react with glycyl part. This shows that Ab formed against the single substance were heterogeneous because they could get Ab with two different specificities. They also found a sizable portion of the Ab that would react only with Ag containing the homologous hapten group, and here they had Ab that required both the leucyl and glycyl groups, which I presume you would say would be the measure of the size of the grouping.

In connection with structures of molecules in Ag-Ab reaction, I think it is very important to realize that the reactions are taking place in aqueous solution. This is part of what Dr. Singer referred to. The Ab is hydrated and the hapten molecules are hydrated also, and the waters of hydration are important parts of the structure. These may well make variations in some of the conclusions that Dr. Kabat has drawn. The sugars are full of –OH groups that can form hydrogen bonds with water and are thus strongly hydrated. The water of hydration may be the important structural feature determining specificity effects between the hapten and antibody.

Then there is also a big factor of orientation, which Dr. Kabat did mention in connection with his heptasaccharide where he proposes a cyclic structure. Actually, all the structures up to the seven-membered sugar can assume a good many different configurations and are probably not extended in an aqueous solution as shown; in which case, Ab formed against a partially curved saccharide may well show different reactions from what one would expect for an extended molecule.

KABAT: With respect to Dr. Pressman's interpretation, of course, we don't know exactly what the shape of the combining site is. I would merely say that this is part of the combining site from our interpretation.

With respect to Dr. Pressman's quoting Landsteiner more accurately than I did, I am afraid I have to differ, although Dr. Landsteiner's paper is available for those of you who want to check. It is perfectly true that when he used the whole complex coupled to protein he got more Ab down, but he could not get down a fraction that had a specificity toward both groups unless the thing was hooked to protein. Now the whole question is really ambiguous.

PRESSMAN: May I quote from Dr. Landsteiner?

KABAT: I am glad this is being recorded, since you are quoting.

PRESSMAN: We don't have a slide here. Dr. Kabat didn't prepare one.

KABAT: Maybe you would like to read my paper, however.

PRESSMAN: I would. It probably would be interesting. Let's take Dr. Landsteiner's compound and put L for leucine and G for glycine. This was coupled to horse serum, or something like that, and injected. Landsteiner then tested the Ab so formed by absorbing with old cell stroma coupled with the material containing only the glycyl group. After he had absorbed the serum with this insoluble material and thus removed all the Ab that would react with a glycyl protein, he absorbed with the similar leucyl compound coupled to an insoluble material. No residual Ab were left that would react with a leucyl protein. After absorbing out both of these so that he could no longer absorb any more, he tested with the glycyl-leucyl protein and obtained a precipitate. The experiment was carried out by absorption on a solid and was rather clean cut.

KABAT: As I remember it he didn't get too much here.

Note added after the Conference. On page 721 under *comment* Landsteiner and van der Scheer ('38) state: "The experiments made with sera for the compounds containing both glycine and leucine (GIL), or succinic and phenylarsenic acid (SA) residues definitely establish the existence of cases in which discrete antibodies are formed that are individually directed towards separate determinant groups in one substance, even though it be of small molecular size and the determinant groups rather closely adjoining. The failure of repeated absorption with an antigen which contains only one of the two groupings, to effect a significant diminution of the antibody reacting with the other group, is proof for the lack of serological kinship between the two antibodies demonstrated." In the next paragraph they further state: "There were practically no antibodies in the sera examined which were not removable by absorption with both 'partial' antigens."

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BONE MARROW TRANSPLANTATION IN ANIMALS EXPOSED TO WHOLE-BODY RADIATION

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The immunologic and genetic features of experiments in which lethally irradiated animals are kept alive by intravenous injection of a normal bone marrow cell suspension after exposure are the subject of this evening's discussion. My brief remarks are intended to provide some background information on the treatment of irradiated animals by this technique.

Basically, the bone marrow experiment is relatively simple (Lorenz *et al.*, '51). A nonirradiated animal is killed and a quantity of blood-forming cells are removed from the bone marrow. The living cells are suspended in buffered saline and injected intravenously into a lethally irradiated isologous animal. In the exposure range immediately above the 30-day LD_{100} dose, this treatment has given 100% 30-day survival in mice and guinea pigs. The treatment is also effective in other species (Fishler *et al.*, '54; Smith *et al.*, '55; Hilfinger *et al.*, '53; Crouch and Overman, '57; Alpen and Baum, '57). Table 1 shows the results of a typical experiment in which isologous bone marrow was injected into lethally irradiated guinea pigs. The radiation dose in the range under consideration leads to acute radiation death because the bone marrow is destroyed.

Considerable work was undertaken in exploration of bone marrow therapy for irradiation injury. It was established that blood-forming tissues are specific in causing recovery.

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[1] Operated by Union Carbide Nuclear Company for the U. S. Atomic Energy Commission.

Adult isologous spleen that contains blood-forming cells, fetal liver, bone marrow, and sometimes whole embryos will cause recovery of the lethally irradiated animal. Adult liver, heart muscle, brain, endocrine glands, and other nonhematopoietic tissues will not promote recovery. The dose of hematopoietic cells necessary for recovery depends on the genetic relation of donor and host and the route of injection (see van Bekkum, this symposium). The route of injection, time of injection (Lorenz and Congdon, '54), and the effect on the LD_{50} 30-day response have been determined (Congdon, '58).

TABLE 1

Effect of isologous bone marrow on mortality of X-irradiated strain-13 guinea pigs

NO. OF ANIMALS	X-RAY DOSE	TREATMENT	30-DAY MORTALITY
	r		%
14	750	Bone marrow intravenously	0
12	750	None	100

The question of mechanism in the bone marrow experiment can be divided into two problems. The first is the prevention of radiation death. This was explained by Jacobson and his group ('51) for spleen shielding and by Lorenz and his associates ('52) for the bone marrow experiment. The prevention of death depends on return of the blood-forming tissues to a normal state. After lethal irradiation, the bone marrow disappears over a period of 2–5 days and remains this way until the death of the animal from hemorrhage, infection, and anemia. There is no new production of granulocytes, and this leads to a precipitous fall in the white blood cells of the peripheral blood. Bacterial infection supervenes and, in some species, is the major cause of death. The platelets reach a critically low level in the blood when there is no new platelet production and hemorrhages occur in many tissues and organs. Comparison of bone marrow sites of treated and nontreated animals showed that the blood-forming tissues returned to normal in a few days in the injected or spleen-shielded animals. This is in marked contrast to the untreated, irradiated animals. With the recovery of the bone marrow, the peripheral blood returns to normal. The white blood cells, the platelets, and the red blood cells have such a short period of depletion that bacterial infection or hemorrhaging does not take place.

Demonstration of the actual mechanism of action of the bone marrow was one of the dramatic developments in the bone marrow experiment. The alternative explanations were outlined by Jacobson for the spleen-shielding experiments ('52). He suggested that the recovered bone marrow could result from some sort of humoral stimulation so that the injured cells would recover, or that it might be a colonization; that is, the recovered cells represent the descendants of the injected cells. Also considered were tiding-over and detoxification theories. It has now been convincingly shown that the bone marrow and peripheral blood cells in the treated animals are derived from the donor cells. This was proved by a variety of techniques.

Lindsley *et al.* ('55) showed that red blood cells of the same antigenic type as those in the rat providing the donor bone marrow appeared in the circulation of treated, irradiated rats. Rat red blood cells were demonstrated by an antigenic technique in the circulation of surviving lethally irradiated mice treated with rat bone marrow (Makinodan, '56; Vos *et al.*, '56). Rat granulocytes were observed in the blood-forming tissues and peripheral blood of surviving lethally irradiated mice treated with rat bone marrow (Nowell *et al.*, '56). A histochemical staining difference was used to demonstrate the rat granulocytic cells. In the same type of "rat-to-mouse" experiment, Smith *et al.* ('57) showed that the platelets in surviving mice behaved antigenically like rat platelets. Ford *et al.* ('56) showed that dividing cells in bone marrow, lymph nodes, and in surviving host mice had chromosomes cytologically resembling those of the donor animal. Antigenic techniques to identify the type of cells in thymus (I. Urso *et al.*, '57;

Merwin and Congdon, '56) and lymph nodes were also carried out.

A problem of great current interest is whether the immune mechanism is transplanted along with the precursors of blood-forming cells. This is particularly important in trying to understand the delayed foreign bone marrow reaction. When isologous bone marrow is given to the irradiated recipient, there is very good 30-day survival at supralethal doses of radiation; and if the animals are observed for a year or so, there may be no additional mortality. Bone marrow, however, from a different strain or from a different species, such as rat bone marrow to a mouse, may give very good 30-day survival; but large numbers of the animals die subsequently (Congdon and Urso, '57).

One of the clearest ways to demonstrate this reaction is to follow body weight. Animals that get irradiation only show progressive weight loss until death, whereas those that get isologous bone marrow will show a temporary weight loss and then a recovery. With foreign bone marrow, there is also an initial temporary weight loss but after recovery starts, a secondary weight loss develops.

This is a rather characteristic response; and as the picture develops, a disease syndrome appears in which the animals, in the presence of excessive weight loss, may still show a normal food intake.

One of the central features of this secondary disease is the lymph node changes. The lymph nodes, and to some extent the spleen, of these animals go through a granulomatous reaction. The granulomatous response results in a more or less fibrotic lymph node that we have intrepreted as a host reaction to the foreign cells. Many plasma cells are present at some stages of the reaction. In the white pulp of the spleen, we frequently find areas of fibrinoid necrosis.

SUMMARY

Recovery from lethal irradiation injury in the bone marrow experiment is caused by a rapid regeneration of blood-forming

tissue that prevents infection, hemorrhage, and anemia. Evidence from chromosomal, histochemical, and immunologic techniques demonstrates that the regenerated blood-forming cells and their products in the peripheral blood are descendants of the injected blood-forming elements. When the donor of the bone marrow is not genetically identical to the irradiated recipient, the transplanted bone marrow can be associated with a delayed immunologic disorder that kills most of the irradiated mice many weeks after exposure.

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FURTHER CYTOLOGICAL OBSERVATIONS ON RADIATION CHIMERAS

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FIVE FIGURES

CBA mice exposed to 950 r of X radiation die within 12 days. Among other changes observed is complete destruction of the bone marrow. Similarly irradiated animals injected intravenously with a suspension of splenic cells from infant mice usually survive for more than 30 days after treatment, the mean survival time being dependent on the strain of mice used as donors. In these recovered animals, the bone marrow is regenerated and the peripheral blood is not detectably abnormal.

Since the latter part of 1955, reports from several laboratories have together demonstrated conclusively that the regenerated marrow is derived from the injected cells, and therefore constitutes, in a sense, a graft. Other workers (Lindsley *et al.*, '55; Nowell *et al.*, '56; Vos *et al.*, '56; Makinodan, '56; Mitchison, '56) employed immunological or cytochemical methods; in our laboratory the approach was cytological (Ford *et al.*, '56, '57). By the use of homologous T6/+ cells carrying a chromosome "marker," it was possible to demonstrate the presence of dividing cells of this type, not only in the bone marrow, but also in spleen, lymph node, and thymus. After a brief initial period during which a few cells of the recipient with radiation-damaged chromosomes were also present, the dividing cells of these tissues were all of the donor's type. Essentially the same result was obtained when heterologous cells from rats were injected. As the time interval between treatment and sacrifice was increased, a sharp difference between the two series became evident. Mice injected with homologous cells retained the grafts; but in animals treated with heterologous cells the grafts were progressively outgrown by cells of the recipient, the extent to which this had occurred seeming to bear no relation to the clinical state of the animal at the time of sacrifice (Ford *et al.*, '57). The homologous cells injected were obtained from the spleens of 6- to 8-day-old mice, and the heterologous cells were from the bone marrow of adolescent rats. It was therefore possible (though unlikely) that the difference in behavior between grafts derived from homologous and from heterologous material was attributable to the disparity either in the type of cell injected or in the age of the donor rather than to the immunological difference. This report will be concerned with the preliminary results of an experiment to test this point, with further observations on our original series of homologous and heterologous chimeras, and with the results of an experiment in which CBA/H mice were exposed to 575 r and then injected with T6/+ cells.

MATERIAL AND METHODS

CBA/H mice were used as recipients in all the experiments and T6/+ mice and Wistar rats as donors. Details of the radiation treatment have been given elsewhere (Barnes and Loutit, '55). The T6/+ mice were from a stock derived from an irradiated male (Carter *et al.*, '55) and were heterozygous for an unequal reciprocal translocation in which one of the two rearranged chromosomes was conspicuously small. They were raised for us by Dr. Mary Lyon.

For cytological examination, Feulgen squash preparations were made after pretreatment of the tissues in hypotonic sodium citrate solution and fixation in acetic-alcohol (Ford and Hamerton, '56). The scoring procedures and the limitations of the method have been discussed elsewhere (Ford *et al.*, '57).

RESULTS

1. *Dose 950 r. T6/+ cells injected.* Batches of 3- to 4-month-old male CBA/H mice were exposed to 950 r of X rays and shortly afterward injected intravenously with suspensions of cells obtained from the spleens of 6- to 8-day-old T6/+ mice. The number of cells injected was not always estimated directly, but hemocytometer counts indicated that it would normally have been about 10^7. The animals were sacrificed at intervals; and preparations made from femoral bone marrow, spleen, lymph node, and thymus were examined cytologically. The results obtained up to 157 days after treatment have already been described but will be summarized here for the sake of completeness. By 5 days after treatment, virtually all the dividing cells examined from the first three tissues, and most of those from the thymus, contained the small T6 chromosome. The chromosomes in the remaining cells showed many structural changes. It is reasonable to attribute the observed chromosomal changes to the radiation exposure and so to indentify the cells containing them as native to the recipient. These cells rapidly decreased in number as the time after irradiation increased, eventually leaving tissue in which (as far as could be determined) *all* the dividing cells were derived from the donated material. There were only two exceptions to this rule; one recipient-type (normal CBA/H) cell was found in lymph node, and another in spleen, from animals sacrificed 28 and 101 days after treatment, respectively. We have now examined four more animals and extended the time interval to 386 days without finding any change in the cytological picture. It would seem that once tissue derived from T6/ + cells is established in CBA/H hosts exposed to 950 r, it persists indefinitely.

2. *Dose 950 r. Rat cells injected.* Similar batches of 3- to 4-month-old CBA/H mice were exposed to 950 r of X rays and injected intravenously with about 5×10^7 cells from the bone marrow of adolescent Wistar rats. The results obtained from animals killed in the first 30 days after treatment gave,

on the whole, a very similar picture to that obtained in the T6/+ experiments. There were, however, two batches in which many normal CBA/H cells were indentified, particularly in the lymphatic tissue, and in which the numbers of structural changes (in cells that showed any at all) were much lower than we had come to expect from previous experience. The reason for this disparity in behavior is not clear. We will consider it exceptional and assume that it is the rule that, by 30 days after treatment, cells derived from the donor have largely or completely replaced those of the recipient. From 30 days onward, we now have results from twenty-five mice. These are presented diagrammatically in figure 1, the information from the separate tissues being combined. Similarly combined data from the individual animals injected with T6/+ cells and sacrificed after 30 days are given in the same figure for comparison.

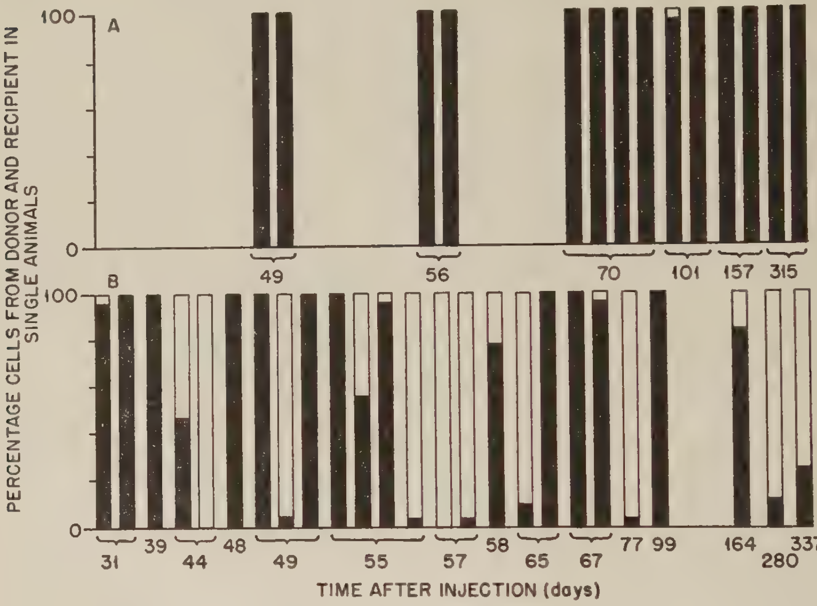


Fig. 1 Chimeras in CBA mice after 950 r of X rays. A: T6 donor; 1890 cells counted. B: Rat donor; 7912 cells counted. Solid bars, donor; open bars, recipient.

This figure demonstrates the point already mentioned that the grafts derived from the injected heterologous cells are eventually replaced by recovering cells of the recipient, the exact time of onset being quite variable. Although the information available is hardly sufficient to warrant generalization, it is our impression that the donated cells, which first appear in bone marrow and spleen and later in lymphatic tissue, also, in general, disappear in the same order. The process of regrowth of the host's own cells would seem to progress rapidly at first but then to slow down and frequently not go to completion. Attention is drawn to the appreciable numbers of cells with undoubted rat-type chromosomes as late as 280 and 337 days after treatment.

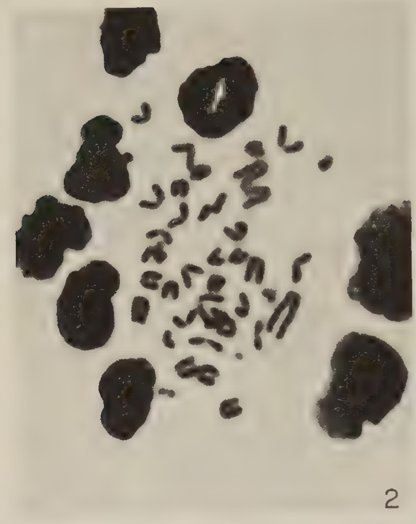
TABLE 1

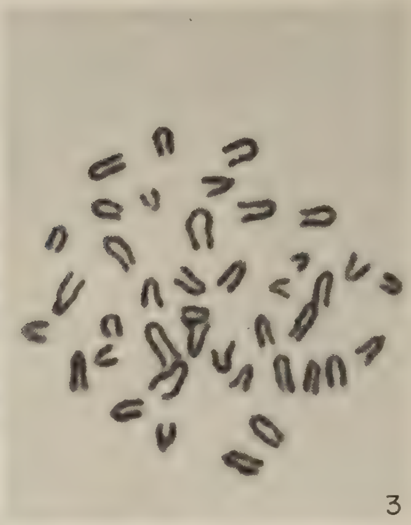
CBA/H mice exposed to 575 r of X rays and then injected with T6/+ cells. Observations on cells in bone marrow and spleen combined

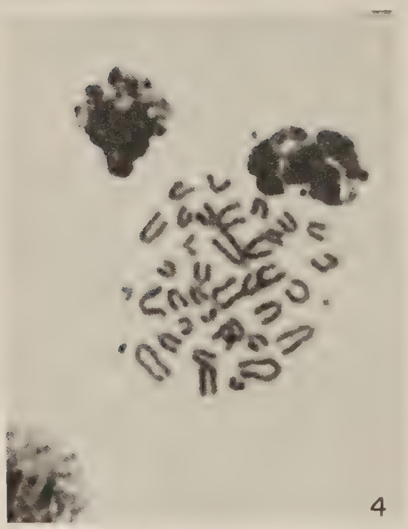
TIME AFTER IRRADIATION	NO. OF MICE	NO. OF CELLS COUNTED		PERCENTAGE OF T6/+
		T6/+	CBA/H	
days				
5	3	343	13	96
11	1	33	98	25
14	3	15	131	10
28	2	8	139	5

3. *Dose 575 r. T6/+ cells injected.* CBA/H mice exposed to 575 r of X rays, but otherwise treated exactly as for the 950 r-T6/+ animals, were sacrificed at 5, 11, 14, and 28 days. Preparations of bone marrow and spleen from a total of nine animals were examined and, since there were no marked differences within groups or between tissues, the data were combined and are presented in table 1. This shows that although the donated T6/+ cells quickly become established, they are rapidly replaced by normal cells derived from the recipient.

4. *Dose 950 r. Comparison of effectiveness of infant spleen and adolescent bone marrow from T6/+ mice and Wistar rats.* As explained in the introduction, the two main groups







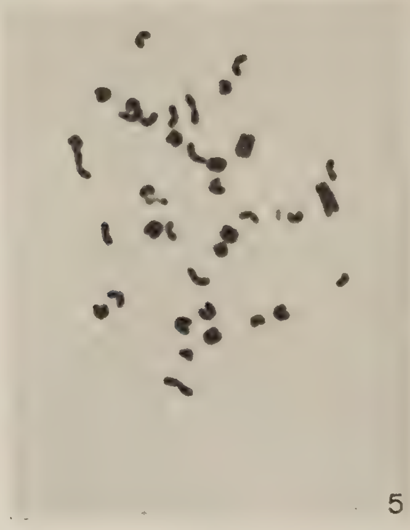


Fig. 2 Cell from bone marrow of mouse 1025 showing the distinctive long and short chromosomes of type-1 cells. × 1750.

Fig. 3 Lymph node cell showing normal chromosomes of the mouse. × 1750.

Fig. 4 Type-2 cell from lymph node of mouse 1025. The two short chromosomes are easily identified. × 1750.

Fig. 5 Cell from spleen of mouse 549 showing two short and two long chromosomes. × 1750.

of treated mice previously considered differered in that cells from spleens of infant mice were injected in the one case (T6/+) and cells from bone marrow of adolescent rats in the other (rat). An experiment was therefore set up to determine whether the age or type of tissue from which the injected cells were obtained would influence the subsequent behavior of the graft. Twenty young CBA/H male mice were exposed to 950 r of X rays and then divided equally into two groups. One group was injected with ca. 2×10^7 cells from spleen of infant Wistar rats and the other with ca. 4×10^7 cells from bone marrow of adolescent rats. This procedure was duplicated a week later, except that T6/+ mice were used as donors and somewhat fewer cells were injected. As yet, results have been obtained only up to 86 days after treatment but is already clear that, as expected, the T6/+ grafts persist whether derived from infant spleen or adolescent bone marrow, whereas there are signs of reappearance of host-type cells in both series of animals injected with heterologous material (table 2).

TABLE 2

Dividing cells from donor (D) and recipient (R) in CBA/H mice exposed to 950 r and injected as shown.
Observations on bone marrow, spleen, lymph node, and thymus combined

SOURCE OF INJECTED CELLS →	MOUSE (T6/+)				RAT			
INTERVAL BETWEEN TREATMENT AND SACRIFICE	Adolescent bone marrow		Infant spleen		Adolescent bone marrow		Infant spleen	
NO. CELLS DERIVED FROM →	D	R	D	R	D	R	D	R
days								
7	106	1[a]	62	2[a]	8	0	18	0
28	97	0	115	0	–	–	–	–
31	–	–	–	–	210	0	323	18
56	50	0	132	0	–	–	–	–
58	–	–	–	–	–	–	285	83
66	–	–	–	–	94	0	–	–
86	233	0	157	0	409	3	343	1

[a] Cells with chromosome structural changes.

5. *Cell clones in vivo.* While examining a preparation made from the bone marrow of a heterologous chimera similar to those described in section 2, a (mouse) cell was found that was characterized by two distinctive chromosomes, one longer than any normal chromosome and one shorter. Since this cell or its progenitor had been exposed to a heavy dose of radiation, it seemed reasonable to suppose that the observed change was the result of a reciprocal exchange of unequal chromosome segments brought about by the radiation. When other cells containing two identical abnormal chromosomes were found, a systematic search was made, with the result that abnormal cells of this kind were found to represent approximately 15% of all the dividing cells. An example is shown in figure 2. The exchanged chromosomes show up clearly in comparison with the normal chromosomes of the mouse shown in figure 3. Similar cells were subsequently found in spleen and in both inguinal lymph nodes, these and the bone marrow being the only tissue specimens taken from the animal in question. In the two lymph nodes, a second abnormal cell type (exchanged type-2; fig. 4) was identified in which at least two and probably four unequal reciprocal exchanges had occurred. One of the two more-striking new short chromosomes was about the same size as the T6 chromosome and the other was considerably smaller. The numbers of normal cells and of abnormal cells of both types in the three tissues examined are given in table 3. The probability of the frequent independent recurrence of the same chromosome exchange must be very small indeed; that of

TABLE 3

Cytological observations on mouse 1025, exposed to 850 r and sacrificed 77 days after injection of rat cells

TISSUE	NO. OF MOUSE CELLS				RAT CELLS
	Normal	Exchanged type 1	Exchanged type 2	Not classifiable	
Bone marrow	57	10	0	0	0
Spleen	76	15	2	10	1
Lymph node	98	56	21	18	0

the same two (? four) exchanges, fantastically low. We conclude that the two abnormal cell types stem from single ancestral cells at the time of irradiation and therefore constitute clones of cells *in vivo.*

Subsequently, similar cell clones were identified in three other heterologous chimeras. As yet, only one of these has been adequately studied. In the spleen, over 50% (65 of 121) of the dividing cells classified had two very distinctive long chromosomes as well as two short ones similar to the short ones just described, the minute chromosome being even smaller (fig. 5). Cells of the same type were identified in preparations of lymph node (the only other tissue available) but the mitoses were too few and the chromosomes not sufficiently well dispersed for quantitative scoring.

The discovery of clones of cells with rearranged chromosomes in four successive animals suggests that such clones may have been overlooked in our previous work. This seems the more likely since the preparations of the earlier heterologous chimeras were scored rapidly (rat chromosomes and mouse chromosomes being so easily distinguished), although it had been appreciated that radiation-induced chromosome rearrangements might be seen in some of the regenerated native cells.

DISCUSSION

It has been reported that T6/+ cells injected into heavily irradiated CBA mice form grafts that persist, whereas injected rat cells give rise to grafts that are eventually outgrown by cells of the recipient. This finding has been confirmed and extended in time, and the rather unlikely possibility that the difference of behavior was influenced by the age of the donor or the anatomical source of the cells injected has been dispelled by the, admittedly incomplete, results reported in section 4.

After 950 r, T6/+ cells persist, yet after a lower dose (575 r) they obtain a temporary foothold but are rapidly outgrown by the recipient's own cells. This indicates that the less heavily irradiated mouse can discriminate between its own cells and T6/+ cells. Now the results with the 950 r heterologous chimeras demonstrate that even after such a heavy dose as this, some viable cells of the recipient remain and are able to multiply to the extent that the rat tissue is very largely or wholly replaced. The type of cell injected after irradiation cannot be supposed to influence the numbers of viable cells available for regrowth. Why, then, are the T6/+ cells in the 950-r homologous chimeras not also replaced by host tissue?

The results presented in section 5 suggest an explanation. The conclusion was inescapable that in one 950-r animal injected with rat cells, over 50% of the dividing cells of the spleen were descended from a single cell at the time of irradiation. This clone of cells was identified by the regular presence of four characteristic abnormal chromosomes. Similar results were obtained in three other similarly treated mice. The evidence suggests that this is likely to be a regular occurrence, and that few clones, perhaps not many more than five or six on the average, contribute to the new growth. Since the chromosomes of the mouse all have nearly terminal centromeres and do not vary greatly in length, many translocations are likely to have passed unnoticed. This is borne out by the finding that only one chromosome (the small T6 chromosome) of the twenty-two involved in the eleven reciprocal translocations produced by Carter *et al.* ('55) was distinguishable in mitotic cells (Ford and Hamerton, unpublished). It is therefore possible that all the regenerating mouse cells in the heterologous chimeras carry chromosome rearrangements. Most are likely also to contain mutant genes. It is therefore suggested that the donated T6/+ cells are physiologically more competent in CBA hosts than clones of (as we suppose) mutated and chromosomally rearranged cells derived from the irradiated host tissue, and that these are more acceptable than cells of a foreign species.

The discovery of mouse cells with a distinguishable chromosome rearrangement — their common origin has been argued – in bone marrow, spleen, and two separate lymph nodes of one heterologous chimera indicates that extensive cell migration can occur from one hemopoietic site to another during regeneration of tissue from the recipient's own cells.

The persistence in many of the long-surviving heterologous chimeras of a small residuum of the donated cells deserves comment. It might be caused by a nonspecific disturbance of the immunological processes as a result of the treatment and the consequent production of a generalized unresponsiveness to antigenic stimuli, or to the acquisition of a state of specific immunological tolerance for cells of the rat. The matter is discussed by Barnes *et al.* (this symposium). The rejection of homologous skin grafts by mice subjected to sublethal doses of X rays but injected with cells from the same donor strain (Trentin, '56) suggests that transient appearance of donated cells in such animals (which is implied by the results of the 575 r-T6/+ experiments) may be insufficient to bring about the same effect.

SUMMARY

It has been reported that persistent grafts of hemopoietic tissue are formed in CBA/H mice exposed to 950 r and injected with T6/+ cells, whereas in similarly irradiated mice injected with rat cells the grafts are ultimately, although perhaps never quite completely, replaced by cells of the host. These results are confirmed and extended in time. Preliminary results suggest that this difference in behavior is unlikely to have been influenced by the anatomical source of the cells or by the age of the donor.

The results of an experiment in which CBA/H mice were exposed to 575 r and injected with T6/+ cells demonstrate that the T6/+ cells appear in bone marrow and spleen but are rapidly outgrown by recovering cells of the host, and by 28 days after treatment have largely disappeared.

In four heterologous chimeras, classes of abnormal mouse cells were identified by the presence of distinctive long or short chromosomes, or both. Each of these cell types is be-

lieved to have arisen from a single ancestral cell in which one or more chromosomal rearrangements had been induced by the radiation. In one case, identical cells were found in bone marrow, spleen, and two lymph nodes. Reasons are given for supposing that chromosome rearrangement may be a frequent phenomenon in the regrowing native tissue of heterologous chimeras that had been subjected to heavy doses of radiation. It is concluded that (1) relatively few ancestral cells contribute to the regeneration of the native tissue in these animals, and (2) migration of cells may take place from one hemopoietic site to another during the process of regeneration.

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TISSUE TRANSPLANTATION IN THE RADIATION CHIMERA

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Mice given a lethal dose of X rays can be induced to recover if treated after the irradiation with an intravenous injection of a suspension of certain normal tissues. The tissues known to be therapeutically effective are: from the mouse — adult bone marrow, infant spleen, and fetal liver; from the rat — adult bone marrow (and from unpublished observations, we would add infant spleen); and from the guinea pig — adult bone marrow. It is notable that all are myeloid tissues.

It has been shown in a number of laboratories (Lindsley *et al.*, '55; Ford *et al.*, '56; Mitchison, '56; Vos *et al.*, '56; Nowell *et al.*, '56) that the recovery is attributable to recolonization of some of the host's X-ray-damaged tissues by the normal cells injected. There seems to be general agreement that the bone marrow is recolonized as a graft of the injected myeloid tissue and that the splenic pulp, which in the mouse (particularly the young mouse) is normally myeloid, is similarly regenerated. Opinions about lymphoid tissue seem to differ. Cole *et al.* ('56) state that "unpublished histologic studies in this laboratory indicate that regeneration of the mouse thymus is not due to direct repopulation by injected bone-marrow cells as such," whereas Ford *et al.* ('56) find the dividing cells in both thymus and lymph glands to be cytologically typical of the donor, not the host. The difference may be only in a matter of words — the italicized words of Cole *et al.*, *as such*. We believe that the cytological evidence of Ford *et al.* reveals the origin of these cells and that the histological appearance in thymus and lymph glands is of regenerating lymphoid tissue (Barnes and Loutit, '56) not of ectopic myeloid tissue. Moreover, in addition to the cytological finding, we have the immunological evidence of Mitchison ('56) that this regenerating lymphatic tissue is of the donor's type. For the present, therefore, we accept the belief that lymphoid tissue is regenerated as a form of metaplasia and myeloid tissue as orthoplasia from the cells injected as therapy. We accept, too, the idea that lymphoid tissue is what is mainly responsible for the immune responses to tissue grafts.

Given these assumptions, we were interested in exploring the immunological responses of animals that had been "lethally" irradiated and induced to recover. In this laboratory, we routinely use the inbred CBA mouse as the experimental subject. With this type of recipient, the best results in terms of longevity are obtained when myeloid tissue from the same inbred strain is given — that is, in the language of tissue transplanters, isologous transfer. If myeloid tissue from mice of a different strain is given (homologous transfer), survival is rather unpredictable. At times CBA mice given myeloid tissue from mice of strain A, although they survive the conventional period of 30 days that indicates recovery from the acute radiation syndrome, die within the next few weeks (Barnes and Loutit, '55): at other times some animals live as long as with isologous transfer. Thus we have had available for test what we may term (1) isologous chimeras, whose tissues are principally derived from the cells with which they were born but whose myeloid and lymphoid tissues we consider to be derived from an identical type of animal, and (2) homologous chimeras, in which the myeloid and lymphoid tissues of a foreign type of mouse are grafted onto the natural background. We have challenged these chimeras with tumors specific to either the CBA or the A strain.

MATERIALS

Sarcoma I. This tumor originally arose in a strain A mouse after treatment with a chemical carcinogen (Dunham and Stewart, '53).

A subline of the tumor was obtained by Dr. N. A. Mitchison of Edinburgh from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, where it has been maintained since its origin in 1948. We in turn received a subline from Dr. Mitchison in December 1955. From the beginning, it grew freely in all strain A mice of this laboratory (A/H), both in the solid and ascites forms. For the first twelve passages ascitic fluid was taken each week from a donor mouse and injected intraperitoneally into another A/H mouse. Throughout this time the tumor was mainly polyploid, according to Ford's cytological evidence.

During exploratory trials the specificity of the tumor was tested. The ascitic fluid (10^6 cells) was injected intraperitoneally or subcutaneously into five strains of mice. Results are given in table 1.

TABLE 1

Mortality of mice given 10^6 cells of strain A tumor, Sa-I

PASSAGE	STRAIN OF MICE AND ROUTE OF PASSAGE						
	CBA/H		C3H/H		C57BL/H		101/H
No. of Sa-1	IP	SC	IP	SC	IP	SC	SC
	Number of deaths/number of mice injected						
2		0/3					
5		1/5		0/5			0/5
6	1/7	0/8	0/5	0/5	0/5	0/5	
7		0/10				0/10	
12	3/5	0/5	2/5	1/5	0/5	0/5	
13		0/6		0/6			
Total	4/12	1/37	2/10	1/21	0/10	0/20	0/5

There was no failure to take and kill in animals of strain A. As expected, there were no deaths among the homologous mice of strains C57 and 101, although the tumors did take and grow. After subcutaneous injection, local masses became visible after one week, increased up to about 1 cm in diameter at two weeks, and then regressed. In CBA and C3H mice, the resistance was apparently weaker, and some deaths occurred, especially when the material was injected intraperitoneally.

In later passages, the ascitic fluid appeared to be losing specificity. The tumor was therefore reselected from the solid phase produced by subcutaneous injection and this process was repeated with increasing frequency until now the practice is to maintain a continuous passage by subcutaneous injection and to prepare an ascitic form for use as a by-product of each passage. *Post hoc*, the cells in the ascitic form are now almost all hyperdiploid (C.E.F.).

Subcutaneous injection of the fluid into the buttock of an A/H mouse leads to the development of a solid tumor that rapidly grows, invades subjacent muscle and connective tissue, and occasionally traverses the peritoneal barrier, giving rise to ascites. The solid tumor extending superficially frequently breaks down and leads to superficial ulceration.

CBA Lymphoma 151/1. The natural incidence of lymphoma in the CBA strain is in the order of 1%, but after irradiation the incidence may approach 100%. It frequently takes the disseminated form rather than the localized thymic form.

The tumor 151/1 arose in one of the irradiated CBA mice of our colleague R. H. Mole ('56a) and has been maintained by passage in normal CBA/H mice. The passage is usually by intraperitoneal injection of a suspension of cells, the suspension being made of electrically minced tissue in physiological saline. Occasionally, the passage was made by intravenous or subcutaneous routes. Spleen and lymph glands were each used as material for passage.

When first examined cytologically in the first-transplant generation, the great majority of the cells contained 41 chromosomes, and this condition was maintained with great constancy in subsequent passages.

This tumor was initially tested for specificity. Being of recent origin, it was, as expected, more highly specific than the sarcoma I, which has a history of several years. The results of passing cell suspensions of this tumor containing 10^6 cells are given in table 2. It has not yet caused death in normal mice except of CBA strain (and F_1 hybrids).

TABLE 2

Mortality of mice given 10^6 cells of CBA lymphoma 151/1

PASSAGE NO. OF 151/1	STRAIN OF MICE AND ROUTE OF PASSAGE									
	C57BL/H		C3H/H		101/H		A/H		Hybrid	
	IV	IP	IV	IP	IV	IP	IV	IP	IV	IP
	Number of deaths/number of mice injected									
2	0/2	0/2	—	—	—	—	—	—	—	—
2	—	—	0/6	0/6	0/2	—	—	—	0/2	0/2
6	—	0/5	—	0/5	—	—	—	0/5	—	—
7	—	—	—	0/5	—	—	—	—	—	—
10	—	—	—	0/15	—	—	—	—	—	—

In the chimeras tested, positive results were scored on death of the animal with tumor or in such severe illness with a progressively growing tumor that the animal was sacrificed to prevent further suffering. A negative result was recorded if there was no progression of the tumor after 6 months.

RESULTS

The tumor 151/1, completely specific for the CBA strain, and sarcoma I (Sa-I), highly specific for A strain, were injected into isologous and homologous chimeras. Table 3 indicates that no CBA mouse, normal or chimerical, survived the challenge with 10^6 cells of the CBA lymphoma 151/1. One

TABLE 3

Mortality of CBA isologous and homologous chimeras and of normal CBA mice injected with CBA lymphoma 151/1 cells. Each injection, 10^6 cells.

TYPE OF MICE INJECTED	PASSAGE NUMBER OF 151/1 AND ROUTE OF PASSAGE							TOTAL
	2 IP	3 IP		6 SC	9 SC	24 SC		
	Number of deaths/number of mice injected							
Isologous chimera,								
CBA + CBA	—	—		—	—	9/9	9/9	18/18
No. of wk established	—	—		—	—	45	10	—
Normal CBA	5/5	5/5		5/5	5/5	5/5		25/25
Homologous chimera,								
CBA + A	5/5	5/5	5/5	4/4	3/3	3/3	1/1	26/26
No. of wk established	4	2	2	3	16	52	44	—

would not expect it in the normal mouse or the isologous chimera. The interest lay in the reaction of the homologous chimera basically CBA but with A-strain lymphoid and myeloid tissue. All these mice died within a matter of weeks from the time of challenge. In the five series of table 3, the times of survival were as follows:

Series	*Survival (days)*				
1	16	18	34	34	51
2	0	23	31	31	45
	34	40	47	50	70
3	21	29	32	33	
4	19	21	24		
5	16	17	17		
	18				

In the normal CBA mice, survival beyond a month is rare. Eleven of 26 mice surviving beyond a month is certainly suggestive of an increased resistance. In two mice, no signs of lymphoma were detected at autopsy, or histologically or, in one of these two, on passage of the spleen. These two probably died from delayed effects of radiation — not uncommon, as we have noted in homologous CBA + A chimeras. In other mice, however, absence of signs of lymphoma at autopsy was shown to be misleading when the animals were examined by test of passage of tissue or histologically, and examples were met where only one of these confirmatory tests was positive. Negative results with all three, therefore, is not conclusive evidence of freedom from lymphoma, even though indicative that lymphoma was not the direct cause of death.

Table 4 shows two things: (1) Isologous chimeras are much less resistant to Sa-I than normal CBA mice. With increasing time after the irradiation and establishment of the chimerical state the resistance seems to increase. Thus, of the 13 animals established for three months and over, only three died from progressive growth of the tumor compared with 15 out of 23 for the animals challenged earlier. (2) Homologous CBA + A

TABLE 4

Mortality of CBA isologous and homologous chimeras and of normal CBA mice injected subcutaneously with Sa-I. Each injection, 10^6 cells.

YPE OF MICE INJECTED	PASSAGE NUMBER OF SA-I									
	2	4	5	7	8	9	12	13	26 + 2	Total
	Number of deaths/number of mice injected									
sologous chimera, CBA + CBA	1/2	4/5 2/5	—	—	—	4/5	3/4 2/5	1/2 1/4	0/5	18/36
No. of wk established	36	6 6	—	—	—	6	7 7	13 13	17	—
ormal CBA	0/3	— —	1/5	0/10	—	—	0/5	0/6	0/5	1/34
omologous chimera, CBA + A	2/2	— —	2/2 3/3	3/3	3/3	—	—	—	3/3	16/16
No. of wk established	36	— —	7 3	3	17	—	—	—	22	—

chimeras were uniformly sensitive to progressive growth of tumor; all the challenged animals, whether long or lately established, died from what appeared clinically and pathologically to be spreading of the tumor locally or generally. In many cases, as well as local expansion of the tumor, there was widespread metastasis to spleen and lymph glands, which were enormously enlarged. The appearances to the naked eye, therefore, were reminiscent not of the classical Sa-I but of a reticulosarcoma.

DISCUSSION

These results illustrate that an X-ray dose of 950 r has, not unexpectedly, a profound influence on the animal. Recovery is probably never complete, even when the animals are treated with isologous marrow. The shortened life span by about a year (Barnes and Loutit, '55) is evidence of this. Similarly, the animals probably never fully recover their immunological responses. In these experiments, after 6 or 7 weeks, when challenged with foreign tumor Sa-I, 15 of 23 mice (65%) were unable to resist, and succumbed. After three months, resistance was greater — three deaths out of 13 mice. This delayed recovery of a near-maximal response is similar to what we have found in sublethally irradiated animals (unpublished observations).

The homologous chimera of strain A tissue donated to the irradiated CBA mouse demonstrates that this procedure confers on the CBA mouse the faculty of "taking" A tumors. Even beyond the three months after the establishment of the state of chimerism, the death rate from Sa-I in a dose of 10^6 cells is 8 out of 8. It is difficult to accumulate large numbers of these animals because the majority die before the end of the third month (*vide antea*). In general, these results of challenging chimeras with a tumor foreign to the host are similar to those of Simmons *et al.* ('57).

The homologous chimeras, like the isologous ones, regularly died after receiving 10^6 cells of the lymphoma of the host's strain (CBA, 151/1). There was a suggestion of some resistance to progressive spread when the lymphoma was given early after the establishment of chimerism; this is when the donated tissue is most hyperplastic (Barnes and Loutit, '56). Subsequently the lymphoid tissue may mature (see Barnes and Loutit, '56; presumably this is representative of the long-lived chimeras) or atrophy and undergo hyalinization (Congdon and Urso, '57; presumably representative of the short-lived animals). However, of the 7 long-lived CBA + A chimeras available more than three months after being established, all succumbed after challenge with 10^6 cells of CBA lymphoma 151/1.

We cannot be dogmatic in our opinion that all the lymphoid tissue of these animals was in fact of strain A origin. We can but infer it from other similar homologous chimeras in which the donor material is chromosomally marked and then 100% of mitoses in lymph tissue are of donor cells at all times (Ford *et al.*, this symposium). This is in contrast to the heterologous mouse and rat chimera, wherein reversion to mouse, according to cytological evidence, took place at various times but was rarely complete in the sample of cells examined. It would appear, therefore, that mouse and rat lymphoid cells can in some instances survive together with what must be an immunological neutrality to each other, occasionally for as long as a year. Our longer-lived homologous chimeras seem to have similar respective neutrality in that they tolerate the growth of tumors of both host and donor. In fact, anything else but neutrality is probably not consistent with survival.

This type of tolerance has been demonstrated also with skin grafts. Firstly, there was the elegant demonstration of Main and Prehn ('55) that irradiated DBA/2JN hosts resuscitated with (BALB/cAnN × DBA/2JN) F_1 bone marrow would subsequently "take" grafts of BALB/cAnN skin. Trentin ('56) showed that other similar chimeras of one strain and its F_1 hybrid would "take" skin from the other parent of the hybrid. He further showed that chimeras of CBA and C3H would "take" both CBA and C3H skin. This is even more impressive than our results with tumors.

Various modifications of immunological response are now well recognized. Normally, of course it would be quite inconceivable for heterologous tissue from a foreign species of animal, or even homologous tissue from a randomly chosen animal of the same species, to survive in a mouse.

Actively acquired tolerance.[1] This type of tolerance for foreign tissue grafts has been reported in animals that have received their primary experience of the appropriate antigen during fetal life. Thus binovular twin calves, which have a cross circulation of blood in fetal life, continue in extrauterine life to produce red corpuscles of two antigenic types (Owen, '45), their own and their twin's. This indicates that the two exchanged and kept erythropoietic cells. Moreover, such twins will accept grafts of each other's skin (Anderson *et al.*, '51). This "tolerance" is acquired naturally. It can also be induced artificially by the injection into fetal mammals and embryonic birds of living cells from animals of the same species (Billingham *et al.*, '56). These examples, it is true, are all of homologous transfer — calf to calf, mouse to mouse, chick to chick. Billingham *et al.*, however, had what they call marginal success with heterologous transfer between duck and chick, and so also have others (Simonsen, '55; Hašek and Hraba, '55).

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[1] Actively acquired tolerance is defined by Billingham *et al.* ('56): "Tolerance represents the specific and systemic failure of the mechanism of immunological response which is brought about by exposing embryos or very young animals to antigenic stimuli." It is difficult to accept their restriction of meaning to a word like "tolerance," which has been used in a wider sense in immunology for generations, but it is fair to be restrictive about a coined phrase like "actively acquired tolerance."

Enhancement. This is a phenomenon seen in its most effective role when the tissue graft is a tumor. Classically, a malignant tumor arising in one strain of inbred mice will not continuously grow in another strain. After a preliminary period of growth, the immune response is invoked in the host, and the tumor regresses. If, however, the host is first conditioned by the injection of lyophilized tumor (that is, killed, but undenatured tumor) or even lyophilized normal spleen or kidney from the same inbred strain of mouse as the tumor, the subsequent implantation of living tumor reveals an alteration of the host's response. Instead of the host's immune reaction leading to the death of the tumor, continued growth of tumor leads to the death of the host (Kaliss, '55). Snell ('54) once considered the enhancing effect to be the same as actively acquired tolerance but Billingham *et al.* have marshalled evidence against this hypothesis. This can be epitomized in their words —"the one represents the effect of a complete antigen on an immature subject; the other, the effect of a modified antigenic stimulus on a system which is fully capable of an immune response." According to these authors, the enhancing effect is much less dramatic when the homograft is normal skin rather than tumor. According to Kaliss, (1) enhancement is specific just as antigen-antibody reactions are specific; (2) the host must be prepared before the tumor is grafted; (3) the responsible agents in tissues or tissue fluids used for conditioning are effective in very small quantities; (4) the effects persist for a very long time; (5) serum from immunized animals (i.e., antibody) acts temporarily as a passive enhancing agent whereas lyophilized antigen leads to active enhancement, actively produced antibody persisting in high titer in the immunized animals for many months (Kaliss, '56b); it possibly acts on the graft inducing an adaptation for growth in a hostile environment (Kaliss, '56a).

Immunological paralysis. Immunological paralysis is a phenomenon of conventional immunization against certain bacterial antigens but not yet identified in tissue transplantation. Felton ('49) showed that, whereas pneumococcal polysaccharide in doses of 0.0005 mg gave an immune response that protected mice against the specific living pneumococci, 0.5 mg altered the reaction so that mice were not protected against the living organism and immunity could not be subsequently induced with the normal immunizing doses. The antigen in fact persisted in these "paralyzed" animals and this persistence of antigen is thought to be related to the paralysis that is specific to the antigen concerned.

Inhibition of allergy. This is another phenomenon of immunology in a field other than tissue transplantation. Guinea pigs can be sensitized to react when drugs such as 2, 4-dinitrochlorobenzene are applied to the skin. Chase ('46) found that prior feeding of this material to the guinea pigs prevented the subsequent induction of the allergy. Such treated guinea pigs could, however, be passively sensitized by the transfer of cells from the peritoneal exudates of actively sensitized animals (Chase, '49).

One thing that these four mechanisms have in common is their specificity. The diminished reactivity of the treated animal is limited to the response to the particular antigen, drug, or foreign tissue that initiated the change. On the other hand, the effects of radiation are nonspecific, and the reaction to many different antigenic stimuli is altered. A similarly nonspecific, diminished reactivity can be seen after the administration of cortisone, and, it appears, zymosan (Herbut and Kraemer, '56).

Bearing these mechanisms in mind, let us consider the radiation chimera again. The radiation has certainly produced initially in the host a nonspecific impairment of response, so that isologous, homologous, and heterologous myeloid tissue may all "take" after intravenous injection. Moreover, in those favorable cases that survive for long periods, this nonspecific tolerance must persist since the graft persists. But the grafted cells produce lymphoid tissue, which, since it has not been subjected to radiation, should when mature react against the host. In the long-lived cases, the grafted lymphoid tissue too must be tolerant. This cannot by definition be actively acquired tolerance; it is not like enhancement or inhibition of allergy. There is, however, a relatively enormous amount of antigen (as host): the conditions are not quantitatively similar to those with which we have previously, in tissue-transplantation, been familiar; namely, a small graft in a large host. It can be postulated that under these atypical conditions one of two things might occur. The host's antigenic material coming to the "foreign" lymphoid tissue might well overwhelm and exhaust it. The lymphoid atrophy and hyalinization, described by Congdon and Urso ('57) and which we can confirm in dead and dying homologous chimeras, could well be the histological expression of the final result. Billingham and Brent ('57) find the same phenomenon in certain mouse chimeras with actively acquired tolerance and Simonsen ('57) has seen it in chicken chimeras. Alternatively, this large quantity of antigenic tissue material might well cause an immunological paralysis of a kind similar to that described by Felton. In this case one might expect the donor-type lymphoid tissue to be anatomically intact (though perhaps quantitatively subnormal) but functionally specifically inert to the host. It should, however, be functionally adequate as far as other antigens are concerned. Since many chimerical animals live for quite long periods of time, one would expect that they could form antibodies against bacterial and similar antigens. The hypothesis would agree with certain unpublished experimental work from this laboratory in which chimeras were challenged with tumors foreign to both host and graft and, in the majority of instances, rejected them. This is similar to Trentin's experience ('56) with foreign skin grafts in chimeras; but it is perhaps more difficult to destroy malignant tissue than normal tissue.

On the basis of this hypothesis, the longevity of a chimera will depend on its functional immunological capacity. An isologous chimera, in which theoretically there should be no incompatibility between host and graft, should manifest neither lymphoid exhaustion nor immunological paralysis; but a failure of the donor cells freely to repopulate the lymphoid system could perhaps mimic lymphoid exhaustion. Rarely, one does meet clinically isologous chimeras showing the syndrome that we have come familiarly to call "homologous disease," death in the second to fourth month after diarrhea and wasting. A similar phenomenon was noted by Mole ('56b), wherein irradiated mice received no therapy but had two or more massive doses of radiation that were together just not lethal at 30 days.

Homologous disease, however, as so far seen by us in its most florid form, occurs where there is a wide theoretical discrepancy between host and graft at the H-2 locus.[2] With the CBA as host, the longevity is progressively impaired as C3H, A, and C57BL tissues are used as donors. Heterologous chimeras in our laboratory usually show the same syndrome in severe form.

We have yet to see a CBA + C57BL chimera survive beyond 3 months. Occasionally, CBA + A chimeras show little or no homologous disease and CBA + C3H chimeras characteristically do well. We have no information about the H-2 locus of other combinations C3H + (C3H × 101) F_1 and (C3H × 101) F_1 + C3H, which also do well. Furthermore, our irradiated CBA mice given myeloid tissue from our hybrid mice containing the T6 chromosome often show no delayed secondary homologous disease. These long-lived chimeras are those with the postulated immunological paralysis.

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[2] Billingham and Brent's "runt disease" seems to obey similar laws.

SUMMARY

Radiation chimeras of the constitution CBA + CBA (isologous) and CBA + A (homologous) were challenged with a CBA lymphoma (151/1) and an A sarcoma (Sa-I).

All animals were killed by 151/1. Sa-I killed all CBA + A chimeras but only a proportion of CBA + CBA. Time since establishment of the chimerical state seemed to be an important factor.

It is postulated that after 950 r of X rays the immunological mechanism of the CBA mouse is nonspecifically inactivated, allowing it to take grafts of homologous and heterologous as well as isologous tissue: the regenerating lymphoid and myeloid tissues are formed from the donor tissue: this confers facilitation for further grafts of tissue compatible with the donor: in homologous and heterologous chimeras, the donated lymphoid tissue may be exhausted by antigens from the host with resulting death of the chimera: alternatively, immunological paralysis may occur with survival of the chimera.

ACKNOWLEDGMENTS

We acknowledge gratefully the very great help we have received from our technical assistants, Misses Janet Hoare and Brenda Burgess, and Mr. M. J. Corp.

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IMMUNOLOGICAL ASPECTS OF HOMO- AND HETEROLOGOUS BONE MARROW TRANSPLANTATION IN IRRADIATED ANIMALS

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SEVEN FIGURES

INTRODUCTION

At present the only effective method of postirradiation treatment of lethally irradiated mice consists in the administration of hematopoietic cells. The observation that protection can aso be achieved by the administration of heterologous (rat) bone marrow has been interpreted to support the hypothesis of a humoral factor being present in the hematopoietic tissue preparations. According to Jacobson ('52), this humoral factor would stimulate the regeneration of the hematopoiesis of the irradiated mice. Through a number of independent investigations, convincing evidence has accumulated to show that the survival of bone marrow-treated mice is attributable to a proliferation of the donor cells that repopulate the hematopoietic tissues of the irradiated host (Lindsley *et al.*, '55; Nowell *et al.*, '56; Ford *et al.*, '56; Vos *et al.*, '56). This is usually explained by assuming that the immunological defense system of the host has been destroyed as a result of irradiation of the total body.

In the present investigation, the effects of homologous and heterologous bone marrow grafts in mice and rats after X irradiation with various doses were studied. The results show that in mice homo- and heterotransplantation is possible only after supralethal doses of radiation to the whole body. The failure of the transplantation after smaller doses of X rays was thought to be caused by the survival of a few antibody-producing cells. We tested this hypothesis by injecting isologous lymphoid cells, together with rat bone marrow cells, into irradiated mice.

Using various homo- and heterologous host-donor combinations, we conducted a quantitative study of the therapeutic effect of bone marrow transplants in irradiated mice and rats. The results indicate that even after supralethal doses of radiation the immunological defense system is not fully paralyzed.

A delayed mortality occurs during the second and third months after irradiation in mice protected with homologous and heterologous bone marrow. This phenomenon was studied quantitatively in large groups of mice, various host-donor combinations being used. The results suggest that antibody production either from the host against the graft or from the grafted tissue against the host may be involved.

METHODS

Animals. The irradiated mice were 8- to 12-week-old males of the inbred CBA and C57BL strains and of the F_1 (CBA males × C57BL females) weighing 20–26 g. Females 6–8 weeks of age of these strains were used as donors of bone marrow and lymphoid cells.

The irradiated rats were adult males of an inbred Wistar albino strain (WAG), weighing 200–225 g. Young females of this strain (100–120 g), 6–8 weeks of age, were used as bone marrow donors.

All animals had access ad libitum to a commercial cubed food. Details on the pre- and postirradiation sanitation of the animals have been presented elsewhere (Wensinck *et al.*, '57).

Irradiation. The mice were irradiated in groups of ten or fifteen in a circular perspex cage. The physical constants of the X irradiation were: 200 kv; 20 ma; filtration, 1.5 mm of Cu; hvl, 1.8 mm of Cu; distance to target, 50 cm; dose rate, 45 r/minute. The dosage was measured on the bottom of the perspex box with a Baldwin substandard ionization chamber. Maximal backscatter was used.

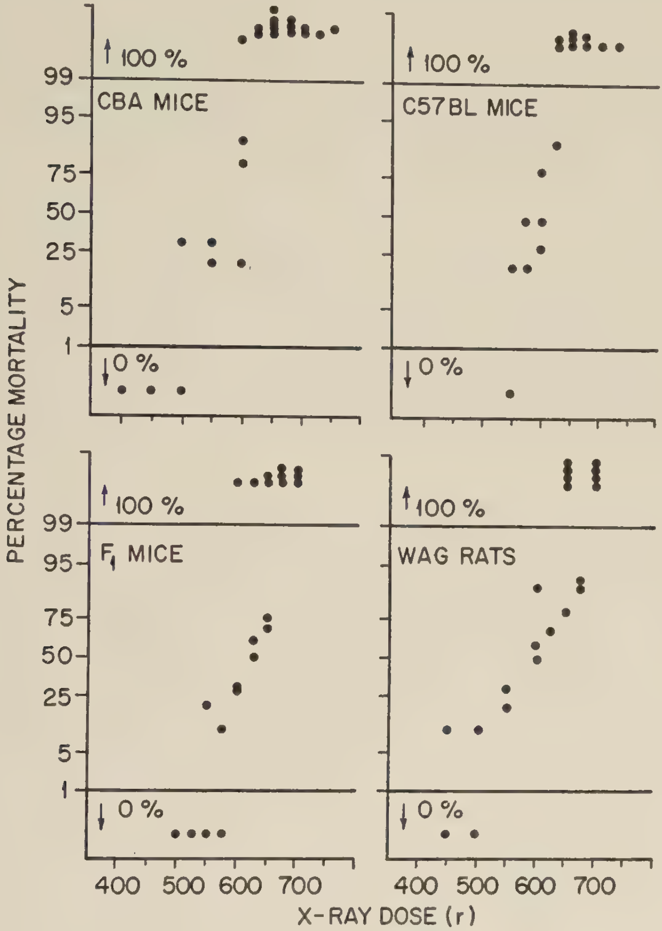

Fig. 1 Relation between X-ray dose and mortality. Points represent groups of 10 animals.

The rats were irradiated in groups of five under similar conditions. The relations between the dose and mortality of both mouse strains, of the F_1 mice, and of the rats are presented in figure 1.

Cell suspensions. Suspensions of hematopoietic tissues were prepared by squeezing the mashed tissue through nylon gauze filters. Suspensions of lymph node and thymus were freed of fat droplets by centrifugation. Bone marrow was obtained from the femurs of mice and rats; for other donor species femurs, tibias, and humeri were used. A hemocytometer was used in estimating the number of nucleated living cells in a 0.2% solution of eosin in Tyrode's solution. The cells that were colored by the eosin were considered to be dead.

Cells were injected into the tail vein (usually in 1/2 ml of Tyrode's solution) within 4 hours after the irradiation, except in a few experiments that will be described separately.

Identification of blood cells. Specific agglutinating antisera[1] were used for differentiation between rat and mouse erythrocytes. Rat granulocytes and mouse granulocytes were differentiated with the alkaline phosphatase histochemical method. Both these techniques have been described previously (van Bekkum *et al.,* '56).

RESULTS AND DISCUSSION

In previous studies, the X-ray doses used slightly exceeded the LD_{100} (Vos and van Bekkum, '56). Under these conditions, homologous and heterologous (rat) bone marrow cells gave quite satisfactory protection to the irradiated mice if enough cells were administered. In the present investigation, the therapeutic effect of optimal numbers of isologous and homologous cells and of rat cells was estimated in CBA mice after various doses of radiation to the total body. The survival at 30 days after the irradiation was taken as a measure of the therapeutic effect. Because in these mice survival is attributable to the proliferation of the donor cells, these values express a relative number of bone marrow takes.

The results are shown in figure 2. As could be expected, isologous bone marrow was effective at X-ray doses up to 1000 r. Higher doses of radiation give rise to the intestinal syndrome that results in death during the first week after irradiation.

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[1] These determinations were kindly performed by Mr. W. W. H. Weyzen of this laboratory.

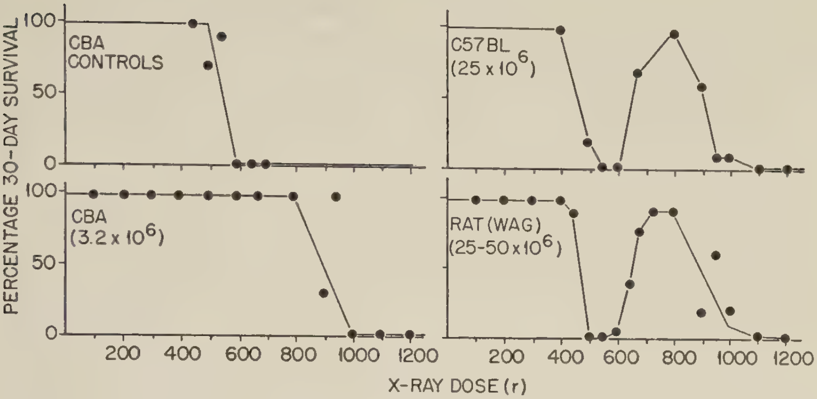

Fig. 2 Therapeutic effect of isologous, homologous, and heterologous bone marrow in CBA mice after various X-ray doses. On each graph the type of bone marrow and the number of cells injected are indicated. Points represent groups of at least 10 animals.

Homologous as well as rat bone marrow cells showed little or no therapeutic effect after X-ray doses of 550–650 r. Moreover, homologous and heterologous cells seem to have an adverse influence at sublethal doses (450–550 r). Only after supralethal doses of radiation up to 1000 r were therapeutic effects observed with foreign bone marrow cells. Similar observations on homologous bone marrow treatment have been reported by Trentin ('56). Gengozian and Makinodan ('56) also found a deleterious effect of rat bone marrow injections in mice after irradiation with an LD_{50}/30 days.

In mice treated with rat marrow, the number of rat erythrocytes and rat granulocytes in the peripheral blood was determined at 11, 35, and 47 days after the irradiation (table 1). Below 650 r, no rat granulocytes were found in the blood. In a few instances, a faintly positive agglutination reaction with specific antirat serum was observed at 11 days, but this may have been caused by erythrocytes that had been injected with the bone marrow.

The radiation doses at which homologous and heterologous bone marrow were ineffective proved to be similar in all cases studied. This observation suggests that the failure of the

TABLE 1

Presence of rat cells in the peripheral blood of CBA mice protected with rat bone marrow

EXPT. NO. AND TIME AFTER IRRADIATION	X-RAY DOSE	NO. OF SUR-VIVORS	ALKALINE PHOS.-POS. GRANU-LOCYTES	ERYTHROCYTES: AGGLUTINATION WITH ANTIRAT SERUM [a]			
				−	+	++	+++
	r		%	No. of mice			
Expt. 1 11 days	950	10	100	0	10	0	0
	800	9	100	1	8	0	0
	675	6	100 (4); 0(2)	4	2	0	0
	600	8	0	8	0	0	0
	500	9	0	9	0	0	0
	400	10	0	10	0	0	0
	300	10	0	10	0	0	0
	200	10	0	10	0	0	0
Expt. 2 11 days	725	10	100	3	5	2	0
	650	10	100	4	6	0	0
	550	7	0	6	1	0	0
	450	9	0	6	3	0	0
Expt. 1 35 days	950	4	100	0	0	4	0
	800	9	100	0	1	8	0
	675	2	92 and 93	0	2	0	0
	600	0					
	500	0					
	400	10	0	10	0	0	0
Expt. 2 47 days	725	5	100	1	0	0	4
	650	2	0	1	0	1	0
	550	0					
	450	8	0	8	0	0	0

[a] −, Only free cells present; +, many small aggregates, nonagglutinated cells more abundant; ++, large aggregates of agglutinated cells, nonagglutinated cells present; +++, large aggregates of agglutinated cells, only a few nonagglutinated cells present.

transplantation is determined by the condition of the irradiated host and not by the origin of the injected cells. It seemed reasonable to assume that in these cases the successful transplantation of the foreign bone marrow is prevented because of the persistence of a functional remnant of the immunological system of the irradiated animals. In view of the extensive destruction of the lymphoid tissues after these relatively heavy total-body irradiations, the number of inact lymphoid cells required to prevent the foreign bone marrow cells from proliferating should be small.

TABLE 2

Effect of isologous lymph node and thymus cells on the therapeutic action of rat bone marrow in irradiated mice

MOUSE STRAIN AND X-RAY DOSE	NO. OF CELLS (× 10^6)[a]			INTERVAL BETWEEN INJECTIONS	NO. OF MICE	
	Rat bone marrow	Mouse lymph node	Mouse thymus		Treated	Surviving 30 days
				hr		
CBA 675 r	42–50	...	...	...	40	22
	42	12.8	...	1	10	0
	42	12.8	...	24	10	0
	..	6.4	...	...	10	0
	50	6.4	...	...	10	0
	50	1.6	...	...	7	0
	50	0.8	...	...	10	7
800 r	25	6.4	...	4	10	0
	25	...	6.4	4	10	0
C57BL 700 to 750 r	All mice of this strain given 26–48 rat cells	...	...	...	19	15
		3.2	...	3–5 for all mice of this strain	10	0
		1.6	...		20	1
		0.8	...		20	0
		0.4	...		20	0
		0.2	...		10	6
		...	12.8		9	0
		...	6.4		20	0
		...	3.2		19	0
		...	1.6		18	0
		...	0.8		10	0
		...	0.4		10	4

[a] Eosin-resistant nucleated cells

This has been confirmed by the results shown in table 2. In these experiments, mice were subjected to supralethal doses of X rays to secure the successful grafting of rat bone marrow and the subsequent survival of the hosts. Either simultaneously or within a few hours after the administration of the rat marrow, isologous lymphoid cells were injected. As few as 4 × 10^5 viable lymph node cells counteracted the therapeutic effect of the rat marrow, showing that the proliferation of the foreign graft can be prevented by a surprisingly small number of intact cells. The injection of lymph node cells 24 hours after the administration of the rat cells was similarly effective. These mice died 10–15 days after the irradiation, similarly to nontreated irradiated mice.

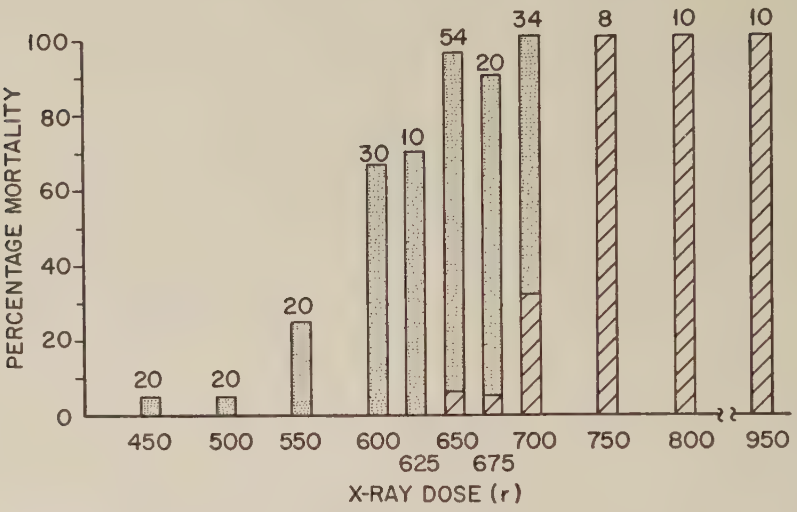

Fig. 3 Mortality of rats after total-body irradiation. Number of rats indicated at the top of each column. Barred columns, rats dying before the fifth day; stippled columns, rats dying beween the fifth and thirtieth days.

These findings indicate that the production of antibodies by the isologous cells and their descendants is sufficiently rapid to cause damage to the foreign marrow cells before their proliferation has proceeded to the extent that enables the host to survive the acute radiation syndrome.

Isologous thymus cells also annihilated the therapeutic effect of rat bone marrow. It is noteworthy that the number of thymus cells required was only little more than that of lymph node cells, although it is generally assumed that the thymus is a rather poor producer of antibodies (Harris and Harris, '56).

In rats the dose range in which death results from failure of the hematopoiesis is much narrower than in mice. Figure 3 shows that at the LD_{100} (700 r) about 30% of the rats die before the fifth day after irradiation, presumably from intestinal damage. After a dose of 750 r, all rats die from the intestinal syndrome.

Isologous bone marrow cells were quite effective after an X-ray dose of 650 r. Minimal protection was provided by 2×10^5 cells; optimal results were obtained with 4×10^7 cells (fig. 4).

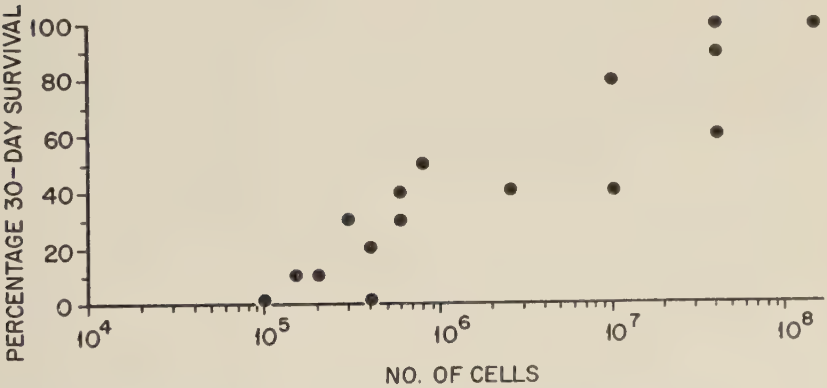

Fig. 4 Therapeutic effect of isologous bone marrow cells in irradiated (650 r) WAG rats. Points represent groups of 10 rats.

Up to 15×10^7 mouse bone marrow cells failed to afford any protection. Considering the favorable effect of rat bone marrow cells in irradiated mice, these observations suggest that an X-ray dose of 650 r does not sufficiently "destroy" the antibody-producing tissues of the rat to permit grafting of heterologous bone marrow. Unfortunately, homologous bone marrow has not yet been tried in this rat strain.

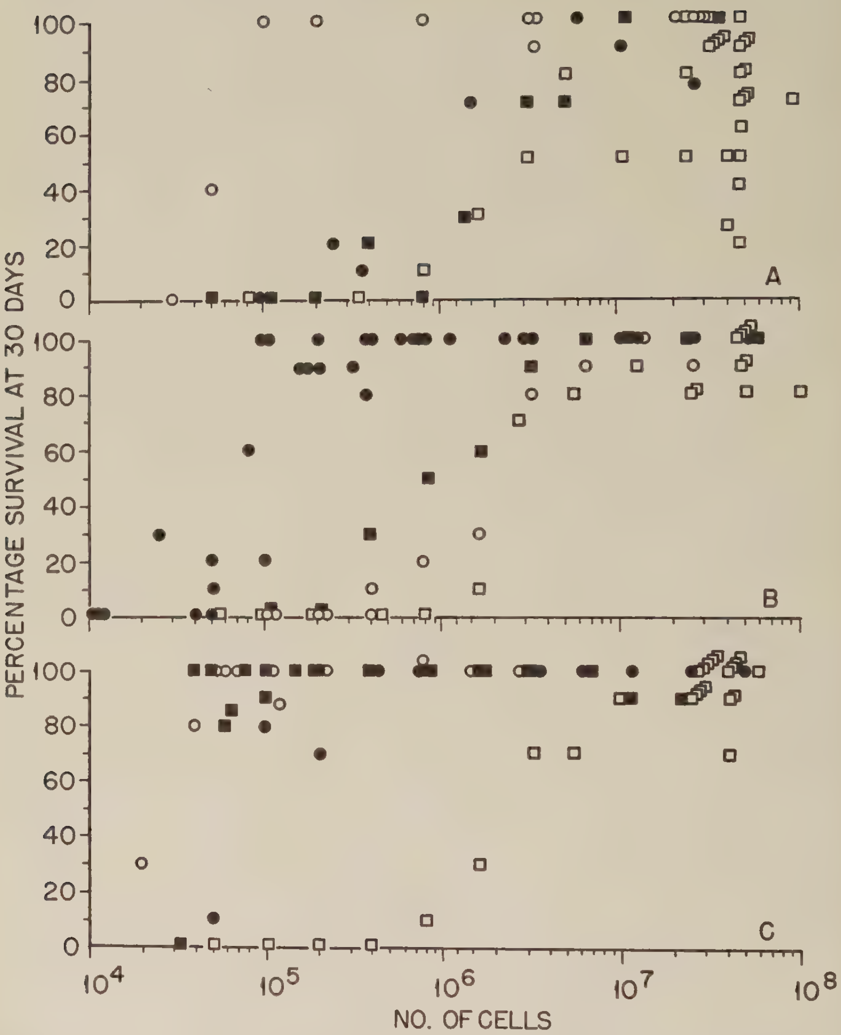

Fig. 5 Therapeutic effect of isologous (same species and strain), homologous (same species, different strain), and heterologous (rat) bone marrow in irradiated mice; in three recipient-donor combinations. *Part A:* recipient, CBA; 675 r. *Part B:* recipient, C57BL; 700 r *Part C:* recipient, F_1; 700–750 r. ○, CBA; ●, C57BL; ■, F_1; □, rat. Points represent groups of 10 animals.

Although a high 30-day survival rate can be obtained in mice after supralethal doses of radiation and treatment with foreign bone marrow, the number of cells required to produce this effect greatly exceeds that of isologous cells. It can be seen in figure 5 that slightly more rat cells than homologous cells have to be injected to produce an identical degree of protection.

Guinea pig bone marrow (24×10^6 cells) increased the survival time in CBA mice (675 r), but all the treated mice died before the thirty-second day after irradiation. Irradiated C57BL mice were not affected by the guinea pig marrow. Bone marrow from Syrian hamsters ($6–10 \times 10^7$ cells) slightly increased the survival time but gave no lasting protection in C57BL mice (750 r); it was ineffective in CBA mice. Bone marrow of adult rabbits and mature rabbit fetuses showed no therapeutic effect whatsoever in both mouse strains.

Figure 5 also shows the results obtained with different host-donor combinations in CBA and the C57BL strains and the F_1 of their cross.

Approximately equal numbers of homologous and F_1 cells are required to produce a comparable protection in C57BL and CBA mice. A much smaller quantity of the parent strain cells will protect irradiated F_1 mice. In fact, the CBA cells are as effective as isologous cells, and C57BL cells seem to be only slightly less effective. These findings agree closely with expectations based on the knowledge of the inheritance of specific tissue antigens in hybrids of inbred strains (Haldane, '54). The available data leave no doubt that the success of the bone marrow transplantation is primarily determined by the degree of antigenic difference between the donor and the irradiated host, suggesting that the production of antibodies is never completely suppressed, even after supralethal irradiation of the total body.

The immunological response that is left, however, can be overcome by increasing the number of grafted cells, provided that the antigenic difference between host and donor is not too great. The explanation of this phenomenon is not evident.

It should be kept in mind that if the bone marrow graft is to prevent the death of the irradiated host, it has to proliferate to a certain minimum size before an irreversible state of pancytopenia develops. This first critical period (acute radiation death) occurs 10–14 days after the irradiation. In the surviving animals, which apparently carry functional bone marrow grafts, some mortality occurs during the next 2 weeks. The mortality in this period is associated with the groups that received relatively small numbers of cells: in these mice, the development of the bone marrow graft appears to be suboptimal. After the thirtieth day, the mortality rate increases sharply in mice treated with foreign bone marrow. This second critical period, which usually lasts until 80–100 days after the irradiation, is known as the period of delayed death (fig. 6). It can be studied best in mice that have received optimal amounts of foreign marrow with regard to the surviving of the first critical period.

The delayed death is preceded by a severe loss of body weight. During the second critical period, most of the mice excrete abundant amounts of loose feces, the condition of the fur deteriorates, and infestation with ectoparasites may become extensive enough to require treatment. In some experiments, many of the dead animals suffered from bacterial lung infections.

The mice that received isologous bone marrow show none of the signs and symptoms of the second critical period.

Figure 6 shows that the frequency of the delayed mortality (here defined as death occurring 30–100 days after irradiation) is very high after treatment with homologous and heterologous bone marrow. However, delayed mortality occurred in only a comparatively small number of F_1 mice after treatment with C57BL bone marrow (fig. 6). Thus, in F_1 mice, C57BL cells behave like isologous cells in the prevention of the acute radiation death as well as of the delayed death. This might be interpreted as suggesting that the delayed mortality is determined by antibody production of the host against the bone marrow graft. This hypothesis, however,

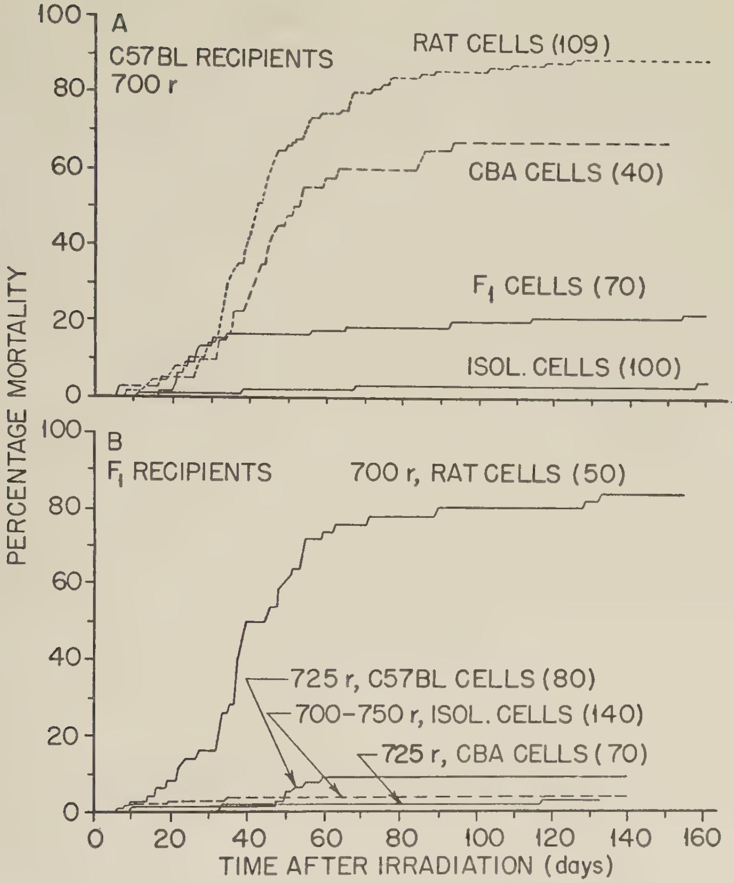

Fig. 6 Delayed mortality in irradiated C57BL and F_1 mice after protection with optimal amounts of bone marrow. Numbers in parentheses indicate number of mice.

also implies that a delayed mortality rate comparable to that observed after protection with homologous cells should occur in C57BL mice treated with marrow from the F_1. Figure 6A shows that this is *not* the case. It should be recalled that F_1 cells behaved like homologous cells in the prevention of acute radiation death of C57BL mice. It should therefore be pointed out that the data presented on the delayed mortality observed in various host-donor combinations can be equally well explained by the assumption that the production of antibodies by the graft determines the death of the animals. At present it is not clear which of the two mechanisms prevails.

The results of skin grafting indicate that, in mice carrying functional foreign bone marrow, the antibody production by the host's own tissues is at a very low level. Main and Prehn ('55) reported that mice protected with marrow from an F_1 hybrid of the irradiated strain subsequently accepted skin grafts from the other parent strain. These results were confirmed by Trentin ('56), who showed furthermore that sucessful homografting of skin is possible in irradiated mice after protection with bone marrow from the same foreign strain as the skin donor. Rat skin grafts were accepted by mice protected with rat bone marrow (Brocades Zaalberg *et al.*, '57). In eleven of twelve mice dying during the second critical period, the rat skin graft appeared healthy, which suggests little activity of the host's own immunological defense system. If an active production of antibodies by the host tissues against the rat bone marrow graft occurred during the second critical period, the rat skin graft would probably show some reaction too.

So far, the results of skin grafting are consistent with the assumption that, in a number of chimeras, antibodies are being produced by the bone marrow graft. In this respect the results of Weyzen and Vos ('57) should be mentioned. These authors demonstrated the presence of rat proteins in the γ-globulin fraction of the serum of mice carrying rat bone marrow grafts. This cannot, however, be considered to prove production of antibodies by the graft, because the rat proteins may be derived from broken-down rat lymphocytes or other rat cells.

On the other hand, there is convincing evidence that some of the surviving host animals regain the ability to produce antibodies even against the foreign bone marrow graft. In the experiments of Brocades Zaalberg *et al.*, three mice failed to take rat skin grafts; in the blood of these animals, rat cells were completely absent. In nine mice, the rat skin graft showed signs of deterioration; in these mice, the host had a mixed population of rat and mouse blood cells.

In a number of mice treated with rat bone marrow, the nature of the erythrocytes and granulocytes has been followed for more than one year. Data on the replacement of mouse erythrocytes and granulocytes by rat cells during the first few months after the irradiation have been presented previously (Cohen *et al.*, '56). The results of long-term studies show four different patterns of the distribution of rat and mouse cells in radiation chimeras (fig. 7). A number of mice survive the second critical period in spite of the rat bone marrow remaining fully active, as shown by the presence of exclusively rat granulocytes and erythrocytes. Other animals dispose of

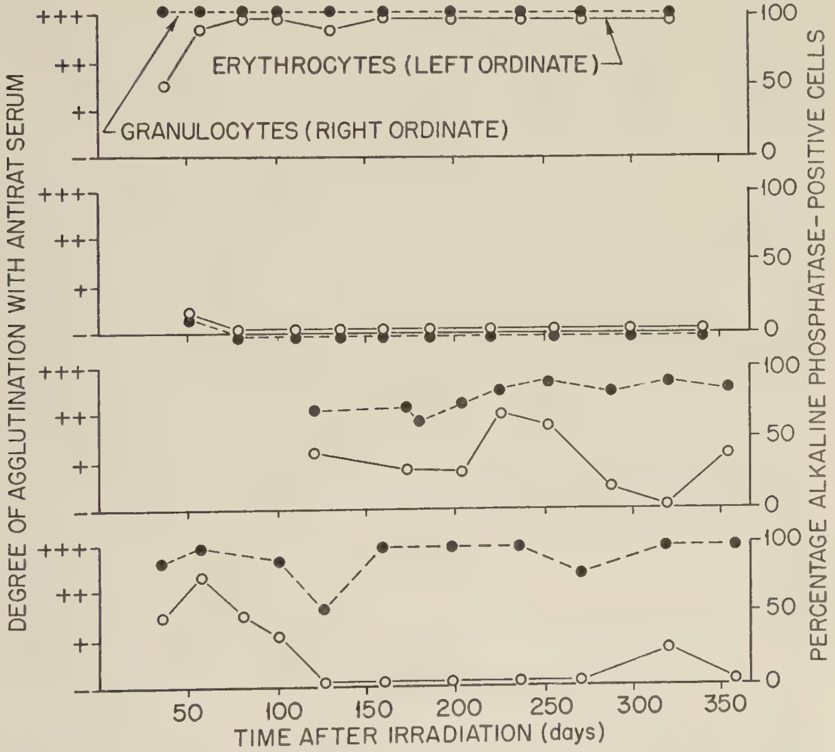

Fig. 7 Nature of erythrocytes and granulocytes in individual mice protected with rat bone marrow. (See footnote to table 1 for explanation of degree.)

the foreign marrow; these are usually called "total reversals." Finally, a number of mice maintain a mixed population of blood cells for long periods. Curiously enough, in some of these animals the erythrocytes were all of the mouse type, whereas the granulocytes were all of rat origin. This indicates that the erythropoietic and myelopoietic systems react rather independently.

SUMMARY AND CONCLUSIONS

The many data accumulated in this laboratory are directly comparable because of the use of a uniform technique and highly inbred strains of animals.

The results of a quantitative study of the effectiveness of isologous, homologous, and heterologous bone marrow show that the number of cells required to protect the host from acute radiation death is related to the degree of antigenic difference between the donor and the host.

If functional grafts of homologous and heterologous bone marrow are to be obtained, the recipients have to be submitted to supralethal irradiation of the total body. Even in these animals, the defense against foreign tissues is not completely inhibited, but successful transplantation can be obtained when sufficiently large numbers of cells are injected, provided that the antigenic difference is not too great. The assumption that some reaction against the graft remains possible, even after relatively heavy total-body irradiation, is supported by the observation that small numbers of intact isologous lymph node cells may prevent the successful transplantation of rat bone marrow in irradiated mice. Although irradiated rats could be protected satisfactorily with isologous bone marrow, no therapeutic effect has been obtained with mouse bone marrow cells, even when large numbers of cells were used. This may mean that the X-ray doses used do not sufficiently inhibit the immunological defense system of the rat.

According to the data obtained on the presence of rat granulocytes and rat erythrocytes in the peripheral blood of mice protected with rat bone marrow, the foreign hematopoietic cells replace those of the host within a few weeks after the transplantation. In some cases, the host's hematopoietic tissues regenerate; this is accompanied by the reappearance of an immunological response against the donor tissues. A state of equilibrium between the host's hematopoiesis and the foreign marrow may be maintained for long periods. In other cases, a complete disappearance of the graft has been observed.

The delayed mortality is virtually absent after treatment with isologous cells. Transplantation of homologous and heterologous bone marrow causes a high rate of delayed mortality. Only a few delayed deaths were observed in F_1 mice treated with parent-strain marrow and also in C57BL mice treated with F_1 marrow. It is considered likely that the delayed mortality is related to immunological reactions. Theoretically, both antibody production by the host against the graft and reversely by the graft against the host's tissues appear possible. It remains to be decided to what extent each mechanism contributes to the pathological condition that results in the delayed death. Additional information, particularly on the lymphoid system of the radiation chimeras, will be required to elucidate this important problem.

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FATE OF INJECTED RAT BONE MARROW CELLS IN IRRADIATED MICE

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FOUR FIGURES

Since the work of Benjamin and Sluka ('08) and Murphy ('14a, b), much has been done in the field of radiation immunology; and on the basis of available data, one would expect that transplantation of any closely related, fast-growing foreign tissue would take place in a lethally X-irradiated recipient. Thus, when rat bone marrow (RBM) is injected into lethally irradiated mice, transplantation followed by the appearance of circulating cells derived from the transplanted precursor cells would be observed. On this premise, we analyzed circulating platelets, red blood cells (RBC), and granulocytes of lethally irradiated mice treated with RBM (950 r-RBM mice) at various intervals.

Using specific mouse antirat platelet and rat antimouse platelet sera, we found that 100% of the circulating platelets were of the rat type in 2–3 weeks (Smith *et al.*, '57). Circulating rat granulocytes, demonstrated by the positive alkaline phosphatase test, were also present (Makinodan, '56). Similar observations had been made by Nowell *et al.* ('56) and Vos *et al.* ('56). Quantitative immunohematologic studies showed that rat RBC first appeared in the blood of these 950 r-RBM mice in one week, and in 4 weeks rat RBC contributed 50% of the circulating RBC (Makinodan, '56). In 9 weeks, 100% of the circulating RBC were of the rat type (fig. 1). Vos and coworkers ('56), in a qualitative immunologic test, also detected rat RBC.

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[1] Operated by Union Carbide Nuclear Company for the U. S. Atomic Energy Commission.

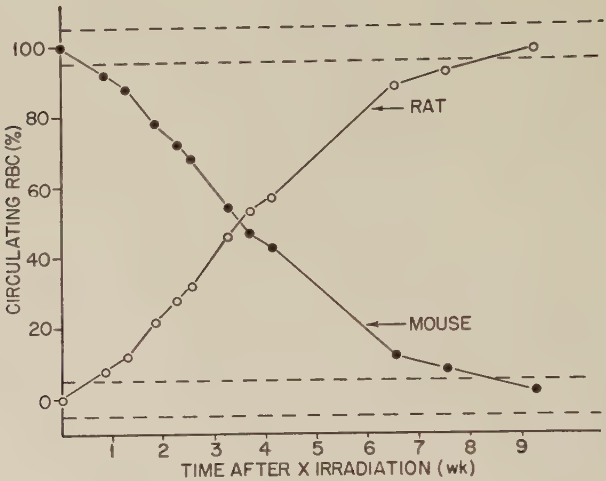

Fig. 1 Appearance of circulating rat RBC in lethally X-irradiated mice injected with rat bone marrow.

TABLE 1

Relative antigenicity of rat-in-mouse RBC

(Mean peak antibody titer $\log_2$)

RBC ANTIGEN (1 ml of 1% suspension)	ANIMALS INJECTED		RBC ANTISERA TESTED			$\frac{\text{AB ABSORBED/UNIT TEST RBC}}{\text{AB ABSORBED/UNIT RAT RBC}} \times 100$
	Normal mice	Normal rats	Rat	Mouse	Rat-in-mouse	
Rat	8.2	0	8.2	0	8.0	100
Mouse	0	7.0	0	7.0	0	0
Rat-in-mouse	8.0	0	8.0	0	8.0	100

To confirm that the RBC of 950 r-RBM mice were of the rat type, other immunological (Gengozian and Makinodan, '56) (table 1) and physicochemical studies (Makinodan and Anderson, '57) were undertaken. The following characteristics of

Isologous thymus cells also annihilated the therapeutic effect of rat bone marrow. It is noteworthy that the number of thymus cells required was only little more than that of lymph node cells, although it is generally assumed that the thymus is a rather poor producer of antibodies (Harris and Harris, '56).

In rats the dose range in which death results from failure of the hematopoiesis is much narrower than in mice. Figure 3 shows that at the LD_{100} (700 r) about 30% of the rats die before the fifth day after irradiation, presumably from intestinal damage. After a dose of 750 r, all rats die from the intestinal syndrome.

Isologous bone marrow cells were quite effective after an X-ray dose of 650 r. Minimal protection was provided by 2×10^5 cells; optimal results were obtained with 4×10^7 cells (fig. 4).

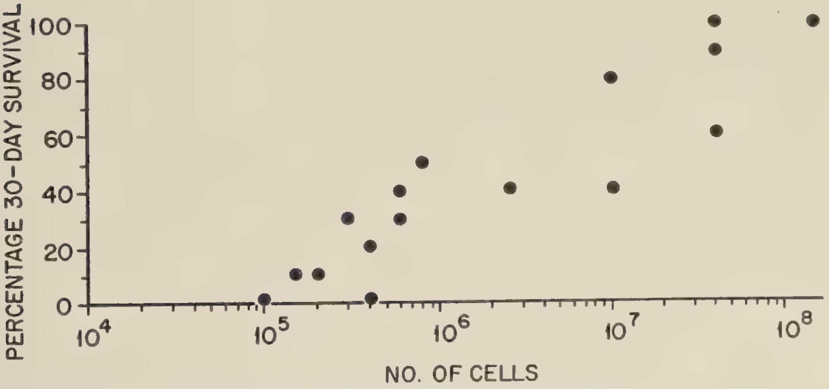

Fig. 4 Therapeutic effect of isologous bone marrow cells in irradiated (650 r) WAG rats. Points represent groups of 10 rats.

Up to 15×10^7 mouse bone marrow cells failed to afford any protection. Considering the favorable effect of rat bone marrow cells in irradiated mice, these observations suggest that an X-ray dose of 650 r does not sufficiently "destroy" the antibody-producing tissues of the rat to permit grafting of heterologous bone marrow. Unfortunately, homologous bone marrow has not yet been tried in this rat strain.

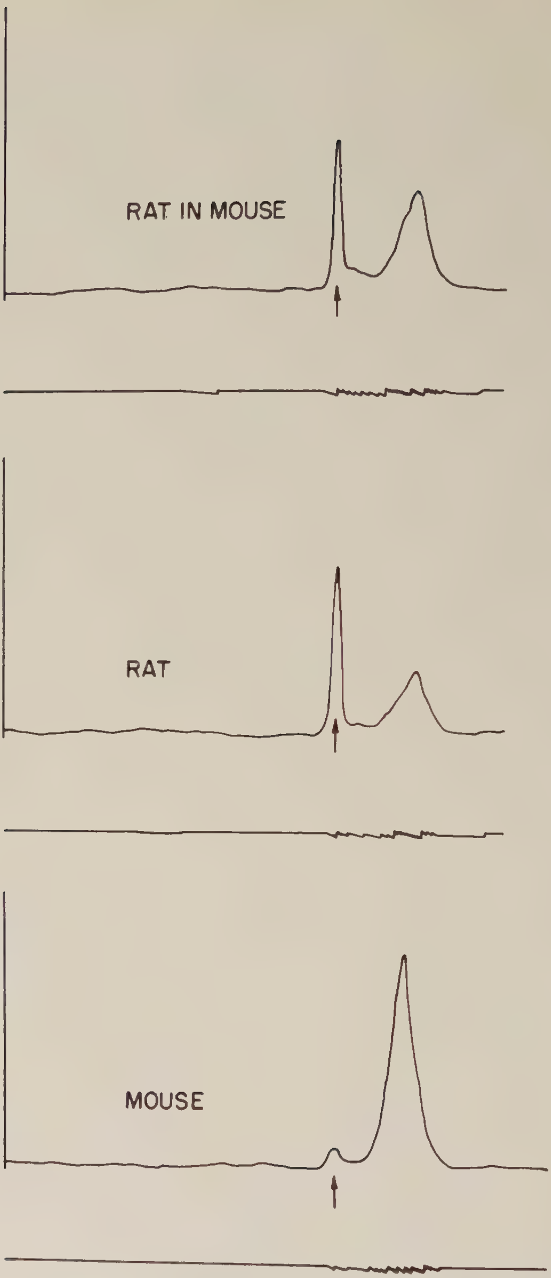

Fig. 3 Electrophoretic pattern of hemoglobin. The arrow indicates point of application of hemoglobin; curve above arrow is pattern of light absorption of paper strip, and curve below arrow is pattern of integrator. (Makinodan and Anderson, '57)

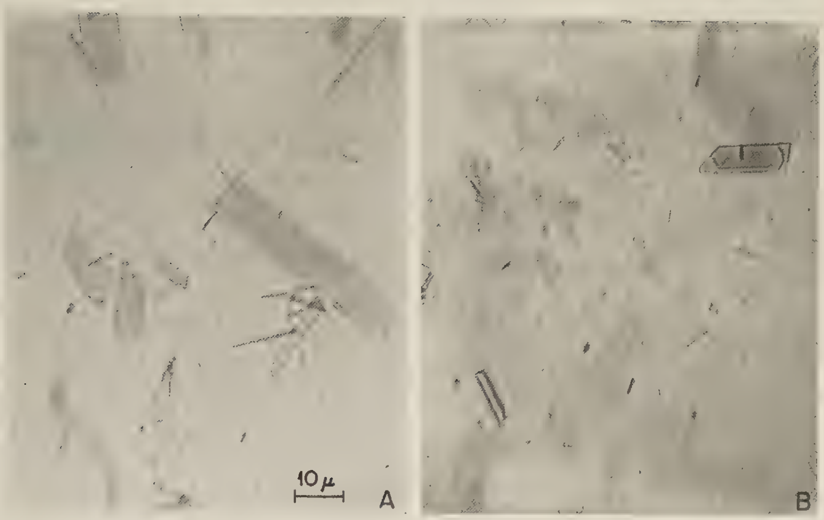

Fig. 4 Photograph of hemoglobin crystals. A, Rat-in-mouse hemoglobin; B, rat hemoglobin. (Makinodan and Anderson, '57)

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GENERAL DISCUSSION ON TRANSPLANTATION OF BONE MARROW

L. J. COLE: One question arises in my mind immediately in connection with Dr. Makinodan's experiments. The range between 710 r ($LD_{25\text{-}30}$) and 1150 r (LD_{100}) seemed to be very wide. This dose-response curve does not seem typical.

T. MAKINODAN: The minimum 30-day LD_{100} for this strain of mice is about 875 r.

COLE: You see no early deaths in the controls at this dose. By early, I mean about the first week.

MAKINODAN: Death takes place 5–15 days after irradiation.

COLE: My point was that, at least in our hands, in this general range of dose, there is a bimodal death response the first part of which corresponds to the ''intestinal mode of death.''

MAKINODAN: I believe it is just the difference in strains of mice.

COLE: Another point I should like to raise is Dr. Loutit's reference to the identification of donated cells in thymus and lymph tissue. From our results using the alkaline-phosphatase technique as a marker for the fate of the injected rat bone marrow cells and also some other attempts to assay the proclivity for cell division of injected thymus cells, it appears that the regeneration or response in the thymus is secondary to the regeneration of the bone marrow. Thus I am not sure that we can speak about the donated thymus cells or lymphocytes, particularly since the injection of thymus cells or lymphocytes alone provides no protection against LD_{100} doses in mice, and also since there is no evidence that the injection of thymus or lymphoid cells in the peritoneal cavity in irradiated mice is followed by multiplication of such cells. Is this a matter of semantics now or is there a fundamental discrepancy here, Dr. Loutit?

J. F. Loutit: I don't think there is any fundamental discrepancy. Ford is looking at nuclei, the chromosomal pattern; you, with the alkaline-phosphastase test, are looking at cytoplasm. We type the dividing cell in thymus as rat-positive on its chromosomal pattern; you say it is phosphatase-negative and we would agree, because lymphoid cells read thus.

Secondly, we would agree with you about time factors. We find significant recovery of marrow at 3 days. At 5 days the marrow cavity is about 50% filled, and at 7 days it is completely full. But the regeneration of the lymph tissue is certainly later, according to the histological evidence. So there is this time difference; the marrow recovers first and then the lymphatic tissue. This may be because the injected material is a mixed crew containing primitive cells of many types, and I don't know which is the effective one (or several) of this mixture. It could be that one of the components recolonizes marrow and another the lymph tissue, but more slowly. Alternatively, the marrow might be recolonized first and seed into the lymphatic tissue.

Cole: There are three points I should like to discuss. First, we found a vast difference between the effect of isologous bone marrow on fast neutron-irradiated mice and the effect on X-irradiated mice. The more striking aspect of this is that the bone marrow is ineffective at doses of neutrons after which the controls die from the early mode of death, and also at doses somewhat lower where the controls die at the usual time—about the third week. We have no reason to suspect that this bone marrow does not "take" in the neutron-irradiated mice. And so to explain why there is no bone marrow protection after fast neutron irradiation, we are suggesting another type of mechanism; namely, a secondary intestinal injury, which becomes limiting to survival even in the face of bone marrow regeneration by the third week, seen in the animals that survive to this time.

Second, we can perhaps make some statements about which type of cell is *not* involved in the protective effect of bone marrow. To illustrate, we know now that in mice in which we elicit hypertrophy of erythropoietic tissue by prior phenylhydrazine treatment, there is no enchancement of the protective effect of splenic homogenates against lethal whole-body X irradiation (Cole *et al.*, Radiology, *64:* 201, 1955). Another type of cell that we have recently attempted to assay with reference to this effect is the large mononuclear leukocyte, presumably macrophage (unpublished studies with G. Santos and M. Fishman). We isolated monocytes from the peritoneal washings of rats 48 hours after injection of proteose-peptone broth and got of the order of 90% of the cell suspension as monocytes. These are rat cells. We find that injecting 70–100 million monocytes intravenously into mice after LD_{100} doses has no protective effect on these animals. Furthermore, these cells give a weak alkaline-phosphatase reaction — weaker than the granulocytes but nevertheless a positive reaction. We find that the alkaline-phosphatase reaction disappears after the second day, so that presumably these cells do not divide any further to give alkaline-phosphatase-positive progeny. We also observe this kind of thing when we inject rat bone marrow into *normal* nonirradiated mice; that is, the initially injected material is seen the first day, but by the third or fourth day the cells have disappeared, so far as the alkaline-phosphatase reaction is concerned.

Third, as Dr. Congdon indicated in the bone marrow experiment, we injected rat bone marrow into X-irradiated mice, and then at various times after irradiation injected the bone marrow of these recipients in turn into a second group of irradiated mice. The same is done in mice that have received isologous marrow, and then their marrow is injected into a second group of irradiated mice. We take advantage of the large differential between the effectiveness, at least in terms of numbers of cells, of rat bone marrow given to mice versus isologous bone marrow. Of the order of half a million cells will protect irradiated mice after a standard LD_{100} dose, whereas you need of the order of 70 million rat bone marrow cells. We have given one million cells as a standard dose at various times after injection of the rat marrow, and also at various times after injection of mouse marrow and found, as one would expect from all the things that we have heard here, that in the mice that have received rat bone marrow injections, at no point up to 36 days is there any effect in terms of protection. In other words, 1 mg of this marrow at all these times does not protect the second group of irradiated mice. If this were mouse marrow, functionally, it would be expected to be effective at a 1-mg dose level at 19 and 28 days but not at 14 days after injection.

C. C. CONGDON: We have worked with fast neutrons (14-Mev) from the Cockcroft-Walton accelerator. Bone marrow treatment after this kind of exposure is about half as effective as after X irradiation. There seems to be some difference between cyclotron neutrons and those observed from the deuterium-tritium reaction of the Cockcroft-Walton.

I want to comment about the lymph nodes. Dr. Loutit has indicated here how the lymph nodes recover after irradiation and, I would say, isologous bone marrow. When foreign bone marrow is given, we do not see this kind of recovery. As a matter of fact, the recovery is qualitatively different at different times. After irradiation, the lymphatic tissue is markedly destroyed, but there is a minimal recovery in the lymph nodes. A few lymphocytes can be found within a day after irradiation. Then the medulla becomes the site of intense granulopoiesis; this occurs with both isologous and with foreign bone marrow. I think that the chromosomes Dr. Ford finds could easily represent dividing cells of the granulocyte series and have nothing to do with lymphopoiesis.

With time, I think somewhere around 20 days, the mice treated with isologous bone marrow show germinal centers and good lymphopoiesis and by 30 days the lymph nodes are back almost to normal, both qualitatively and quantitatively. This is not the case with foreign bone marrow. We see the granulopoiesis and granulomatous response during the early stages, but the lymphopoiesis does not return in many animals or only to a small degree. We have seen at late intervals some animals, as Dr. Loutit has, that received foreign bone marrow and did have a relatively normal-looking lymphatic system. This same kind of change can take place in the white pulp of the spleen, sometimes being only a matter of extreme atrophy of the lymphatic tissue of the spleen and other times leading to fibroid necrosis.

The thymus, after foreign bone marrow, recovers partially by the twentieth day, then degenerates again. Then animals that survive or live longer with the foreign bone marrow reaction may recover again. Applying specific antisera to thymocytes, Gengozian and Urso found that at the early stage of regeneration the thymus was of the mouse type and at the last stage of regeneration it was of the rat type.

LOUTIT: I would like to invoke here the possibility that the difference between us is the question of strains; we do not work with identical material. That is one explanation. Another, I am sure, is variation, even within our laboratory, with time. What I was quoting was an experiment including homologous and isologous chimeras examined at the same time; you just cannot distinguish between them. But I am convinced that it just happened to be one of those favorable periods. If we had done the same experiments at another time we might well have gotten your results, because we certainly see the same end result although we have not obtained it with serial sacrifice.

I. R. SCHWARTZ: Although I have not done any particular work in trying to elucidate the type of antigen-antibody reaction, I am sure that there are two types of reactions occurring in these animals. Two types of reticuloendothelial systems exist in these animals, and the chances are that the antibodies produced by one reticuloendothelial system are attacking the reticuloendothelial cells of the other system in these chimeras. In the long run, probably one of three things happens: the host reaction wins out, the graft response wins out, or they both manage to live in the same house together in a sort of coexistence. In these homologous reactions, I think there is some evidence here on both sides. We find on one side of the fence that the good state of the bone marrow and the lack of a peripheral depression in the blood seem to indicate that the graft situation is faring quite well, while the animal tissue is faring quite badly. I cite the lack of anemia and the still fairly good reserve condition of the bone marrow as some evidence in favor, shall I say, of the graft versus host reaction.

Dr. Makinodan's inability to find rat γ-globulin in irradiated mice treated with rat marrow may result from the overwhelming amount of mouse antigen present, which absorbed the antimouse protein (rat protein), so that it was not easily discernible by ordinary methods in the blood serum. That this antibody may, indeed, exist is brought out by Dr. van Bekkum's work, in which he showed that mouse skin grafts put on mice that had been treated with rat bone marrow will be rejected. In other words, the isologous graft was rejected, and that rejection was probably through an immunological mechanism that necessitated the presence of an antibody against the mouse material. The tumor that Dr. Loutit presented did not do that, but that may be because the tumor is malignant tissue and may be able to break through this immunological barrier more easily than normal tissue, such as skin.

As to Dr. Makinodan's example of a shift in the time of radiation death after lowering the X-ray dose, I wonder if that death is comparable to the death called the "delayed reaction death," or whether it is similar to that described by Dr. Ford where the lower doses produced an incomplete destruction of the immunological mechanism so that this graft never had a really good chance to take hold. In other words, was this kind of death really an acute radiation injury caused by lack of bone marrow proliferation rather than a true delayed homologous type reaction as has been described?

CONGDON: Well, I think there is good histological and histochemical evidence that when 710 r is given to the (C3H

× 101)F_1 mouse, rat bone marrow takes and then is rejected. A plot of the amount of bone marrow in histologic sections with time after irradiation shows that 710 r profoundly depresses the cellularity and it stays quite low, but gradually recovers by 30 days. Now if we give isologous bone marrow to the 710-r animals, there is the same initial depression followed by a very rapid recovery. Rat bone marrow gives the same picture initially. It may even recover faster, but then within 2 days, the rat bone marrow is wiped out and the animals die of anemia, hemorrhage, and infection in 8–16 days. We can reverse this by giving isologous bone marrow at a critical point; the animals live.

The spleen in the 710-r mice treated with rat bone marrow shows the same changes; that is, the red pulp of the spleen will fill up with the rat bone marrow. Then within a day's time, the entire rat complement is wiped out and is replaced by a marked reticuloendothelial proliferation.

D. W. VAN BEKKUM: Dr. Schwartz, we did not transplant isologous skin in the chimeras that received rat bone marrow and rat skin; it was homologous skin. But I should add that your conclusion may still apply. Trentin and also Main and Prehn have shown that irradiated mice treated with F_1 bone marrow accept skin grafts of the other parent strain that was homologous to the host. Therefore, in this particular situation, the host does not seem to be able to produce a reaction against the homologous skin graft. Since in our rat bone marrow-treated mice, the homologous skin graft *was* rejected, it is possible that this rejection was caused by the rat bone marrow graft.

With regard to the demonstration of rat proteins in these mice, I should mention that Dr. Weijzen and Dr. Vos in our laboratory have demonstrated the presence of rat proteins in the serum of rat bone marrow-treated mice. They were *not* able to obtain sufficiently high titers upon using mice and rats to produce antirat and antimouse serum, respectively, but they succeeded when using sera obtained from chickens that had a very high titer. I should add that these sera were highly specific, indeed, and that the determinations were performed on mice that showed exclusively rat type cells in the peripheral blood. The mice under discussion were examined about 100 days after the irradiation and treatment with rat bone marrow cells. The presence of rat serum proteins in these mice could be demonstrated beyond any doubt and, furthermore, this rat protein component travels with the γ-globulin fraction in the microimmunoelectrophoresis experiment.

P. C. KOLLER: Dr. Loutit showed in his pioneering work that by using transplantable tumors he could explore the immunological specificity of chimeras. The preliminary results were encouraging, and it was therefore a pleasure to accept Dr. Loutit's invitation to join him and his colleague, Dr. P. L. T. Ilbery, at Harwell with the purpose of carrying further the investigation of tumor transplantation in the chimeras.

The first idea that occurred to us was to find out if we could transfer the immunological specificity of the donor mouse to the irradiated host. As a first step toward this aim, we made isologous chimeras, using CBA strain as host and donor; the donor material consisted of bone marrow in one set of experiments and in others bone marrow to which were added cells from homogenized lymph nodes. The isologous CBA/CBA chimeras were challenged subcutaneously with 1×10^6 cells of sarcoma I. Dr. Loutit found that this tumor takes and grows in certain proportion of CBA/CBA chimeras given bone marrow. In our experiments, sarcoma I has also taken in those CBA/CBA chimeras that were given cells from both bone marrow and lymph glands. The only difference we observed was a qualitative one; sarcoma I regressed more rapidly in those chimeras that received lymph glands than in those receiving only bone marrow. This was particularly true when the donor was immunized against sarcoma I. In experiments now in progress with BALB/c isologous chimeras being challenged with several tumors of other strains, we are exploring further the immunological response of chimeras that were given cells from bone marrow with or without cells from lymph glands or spleen. The results obtained so far seem to suggest that the recovery of the immune mechanism in the irradiated host is enhanced by the addition of cells from the lymph nodes or spleen.

In another set of experiments semi-isologous chimeras were used. Crosses were made between mice of strains A and C57BL. Immunologically, the two strains are very distinct. A has H-2^a, C57BL has H-2^b antigen. The parental strains, A and C57, were immunized; thus A mice were immunized against 138/1 lymphoma of C57BL, and C57BL mice were immunized against sarcoma I. These immunized mice were used as donors to the F_1(A × C57BL) host and the chimeras were challenged with the tumor against which the donor was immunized. The small number of chimeras that survived the ''secondary'' disease shows that immunity was not transferred through the donor. On the other hand, when mice of one of the parental strains were X-rayed and given F_1 (A × C57BL) donor material (bone marrow without or with lymph node), the chimera became tolerant to the tumor of the other parental strain; thus sarcoma I has grown on C57BL/F_1 (A × C57BL) chimera. In this chimera, the host is C57BL, which is known to be completely immune to sarcoma I. Similarly, the C57BL lymphoma could be grown on A/F_1 (A × C57BL) chimera, in which the X-rayed host was of A strain. Trentin used transplantation of skin to test the immunological behavior of chimeras and made similar observations to ours; he was able to transfer successfully the F_1 hybrid skin to the parental strains.

THE ACTIVATION OF AMINO ACIDS AND CONCEPTS OF THE MECHANISM OF PROTEIN SYNTHESIS

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FOUR FIGURES

In considering the enzymic mechanisms by which proteins are synthesized by living cells, problems are encountered that are unique to protein synthesis and have not been met with in considerations of the biosynthesis of other types of molecules. This is a consequence of proteins being constructed from a number of different amino acids, which are apparently arranged in a specific sequence, depending on the particular protein and the cell or species in which it is made. Furthermore, the specificity of proteins, whether considered as enzymes, hormones, or antigens, is maintained from generation to generation—making it clear that in some manner this specificity is under genetic control. Thus we have to consider, for the first time, enzymic mechanisms for the construction of specific molecules wherein the sequence of the building units, rather than being random as with glycogen and cholesterol, are under quite rigid control. One mechanism that can account for exact replication of specific protein molecules is the familiar template theory, first proposed by Breinl and Haurowitz in 1930 for antibody formation and generalized by Haurowitz in 1950 to include all protein synthesis. This theory is extremely attractive and convenient because it is general enough to allow us to fit new facts onto its framework as they arise. Several attempts have been made to formulate in more precise chemical terms the nature of the template and how it might act (Koningsberger and Overbeek, '53; Dounce, '51-52; Lipmann, '54; Borsook, '56). The mechanism by which the template may function has invited considerable speculation, especially since experimental findings that might impose restrictions on such speculation have been quite scanty. We should also like to examine some of the experimental observations that should be taken into account in speculations about mechanisms of protein synthesis.

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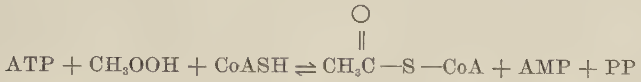
[2] Operated by Union Carbide Nuclear Company for the U. S. Atomic Energy Commission.

Some questions pertinent to the mechanism of protein synthesis are: (1) What structure or structures carry information into a cell and determine the specificity of proteins? (2) How much or how little information is necessary to construct a unique protein? (3) What is the nature of the template or organizer and how does it act? What are the roles of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in protein synthesis? (4) What are the chemical steps that describe the details of the events that take place between the free amino acids and the final protein?

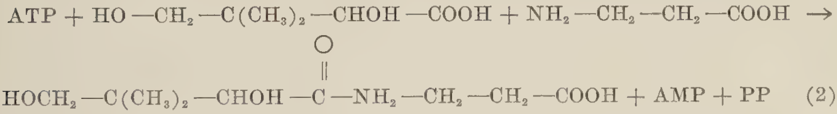
Since proteins are constructed from some twenty units, enzymic mechanisms for bringing about this synthesis would be extremely complex were it not that only one type of covalent chemical bond is used in fabricating the peptide backbone of a protein molecule. Thus as a first simplification we need only to consider mechanisms that bring about a condensation between the carboxyl group of one amino acid with the amino group of another, and, as far as reaction mechanisms are concerned, this leads to a fairly simple consideration.

From a study of model systems for peptide bond formation, such as hippuric acid synthesis (Cohen and McGilvery, '47; Chantrenne, '51), glutathione (Bloch, '49), and the acylation of aromatic amines (Lipmann, '45), came the idea that a reasonable first step in protein synthesis would be the activation of amino acids by interaction with adenosinetriphosphate (ATP). Unfortunately, the activation stages in these model systems proved to be too specific and not generally applicable to protein synthesis. The study of the synthesis of acetyl-coenzyme A by Lipmann and his colleagues, however, provided a clue to a possible mechanism for the general activation of amino acids. When acetate, ATP, and CoA (coenzyme A) are allowed to interact with an enzyme derived from pigeon liver, or from yeast (Lipmann *et al.*, '52) acetyl-CoA is formed by a process involving a pyrophosphoryl split of ATP, as shown in equation (1).



$$\mathrm{ATP} + \mathrm{CH_3OOH} + \mathrm{CoASH} \rightleftharpoons \mathrm{CH_3}\overset{\overset{\displaystyle O}{\|}}{\mathrm{C}}-\mathrm{S}-\mathrm{CoA} + \mathrm{AMP} + \mathrm{PP} \qquad (1)$$

This reaction represents a new type of cleavage of ATP in which the middle high-energy phosphate bond instead of the terminal one is utilized. Since this reaction is freely reversible, the use of PP^{32} (radioactive pyrophosphate) would result in the incorporation of isotope into ATP. The use of PP^{32} presents a simple method for following this type of reaction. Somewhat later, a similar pyrophosphoryl split of ATP was shown to occur in the synthesis of pantothenic acid by a purified enzyme from *Escherichia coli* (Maas and Novelli, '53).



$$\mathrm{ATP} + \mathrm{HO}-\mathrm{CH_2}-\mathrm{C(CH_3)_2}-\mathrm{CHOH}-\mathrm{COOH} + \mathrm{NH_2}-\mathrm{CH_2}-\mathrm{CH_2}-\mathrm{COOH} \rightarrow$$
$$\mathrm{HOCH_2}-\mathrm{C(CH_3)_2}-\mathrm{CHOH}-\overset{\overset{\displaystyle O}{\|}}{\mathrm{C}}-\mathrm{NH_2}-\mathrm{CH_2}-\mathrm{CH_2}-\mathrm{COOH} + \mathrm{AMP} + \mathrm{PP} \qquad (2)$$

This reaction is essentially irreversible. It was subsequently shown, however, that in the presence of pantoic acid alone PP exchanged rapidly with ATP (Maas, '56), suggesting that the first step in the reaction involved the activation of the carboxyl group of pantoic acid. Because the synthesis of pantothenate involves the formation of a pseudopeptide bond, Lipmann postulated a general mechanism for the activation of amino acids for protein synthesis, using the new type of cleavage of ATP (Lipmann, '54). This hypothetical scheme is illustrated in figure 1.

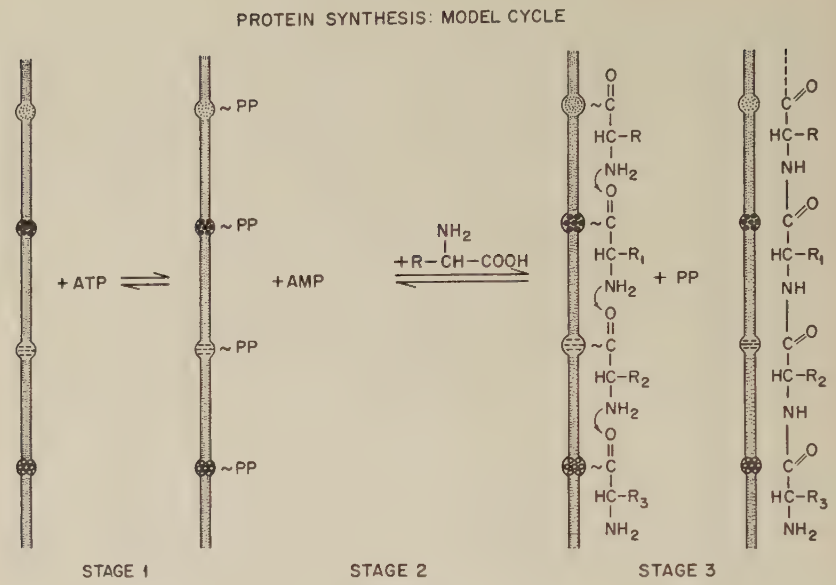


Figure 1.

In this scheme it is assumed that there is in the cell a structure (the nature of which is not specified in the hypothesis) that has on its surface activation sites specific for certain amino acids. In stage one, the various centers interact with ATP, causing a transfer of the pyrophosphoryl group from ATP to the specific sites, leading to their activation. In stage two there is an exchange of pyrophosphoryl groups with the carboxyl groups of specific amino acids whose nature is determined by the specific surface. This process results in the activation of the carboxyl groups of the amino acids. Stage three represents the spontaneous condensation of the activated amino acid into a polypeptide chain (peptide polymer) and the removal of the newly synthesized protein from the specific surface, leaving the surface free to act again.

Somewhat earlier similar theories for describing protein synthesis were proposed (Dounce '51-'52; Koningsberger and Overbeek, '53; Borsook, '56) differing from this scheme in a number of particulars, especially in that the latter theories specify the specific surface as RNA and the sites as the phosphate groups. We have chosen to present Lipmann's scheme, because, when it was proposed in 1954, it suggested an experimental approach to the problem that has since borne fruit.

Careful examination of the scheme reveals that if the events depicted in stages one and two are reversible, they should lead to an exchange of the pyrophosphate moiety of ATP with P^{32}-labeled inorganic pyrophosphate *only* in the presence of amino acid.

Using this approach to the problem, Hoagland discovered in rat liver extracts an amino acid-dependent exchange between inorganic pyrophosphate and ATP (Hoagland, '55; Hoagland, Keller, and Zamecnik, '56). A similar exchange reaction was found to be ubiquitously distributed among microorganisms (DeMoss and Novelli, '55a, b).

When a crude extract of *E. coli* is incubated with ATP and P^{32}-labeled PP, there is a rapid exchange of PP with the pyrophosphoryl moiety of ATP, which is stimulated by the presence of amino acids (table 1). That the exchange reaction occurs only with intact PP, without its prior hydrolysis to orthophosphate, is evident from the lack of exchange when P^{32} orthophosphate is used in place of PP. Thus it appears that the exchange reaction is dependent on both PP and a variety of amino acids.

TABLE 1[a]

Requirements for PP-ATP exchange reaction in Escherichia coli extract

MATERIAL	SPECIFIC ACTIVITY
	cts/min/μmole of ATP
Extract + PP^{32}	538
Extract + PP^{32} + amino acids[b]	1590
Dialyzed extract + PP^{32}	135
Dialyzed extract + PP^{32} + amino acids[b]	1320
Extract + P^{32}	11
Extract + P^{32} amino acids[b]	15

[a] From DeMoss and Novelli ('56).

[b] Nineteen amino acids.

This reaction is widely distributed among all genera of bacteria examined (table 2). The distribution of the exchange activity in several organs of the rat is shown in table 3. The reaction is present in bone marrow, chicken erythrocytes, and a variety of plant materials (G. D. Novelli, J. W. Davis, and A. N. Best, unpublished). In short, it appears to be a universally distributed property of living tissue.

TABLE 2

Distribution of PP-ATP exchange reaction in microorganism [a]

ORGANISM	RATE OF PP EXCHANGE	ORGANISM	RATE OF PP EXCHANGE
	µmoles/hr/mg of protein		*µmoles/hr/mg of protein*
Desulfovibrio desulfuricans	0.8	*Proteus vulgaris*	8.1
Rhodospirillum rubrum	1.8	*Neurospora crassa*	8.9
Leuconostoc mesenteroides	2.5	*Staphylococcus aureus*	12.2
Azotobacter vinelandii	4.1	*Escherichia coli*	15.0
Baker's yeast	4.9	*Serratia marcescens*	15.1
Clostridium butylicum	5.3	*Aerobacter aerogenes*	25.2
Micrococcus lactilyticus	7.6	*Streptococcus hemolyticus*	41.2
Clostridium pasteruianum	8.0		

[a] From DeMoss and Novelli ('56).

TABLE 3

Distribution of PP^{32}-ATP exchange in rat organs

ORGAN	RATE OF PP^{32} EXCHANGE	ORGAN	RATE OF PP^{32} EXCHANGE
	µmoles/hr/mg of protein		*umoles/hr/mg of protein*
Liver	2.6	Spleen	0.9
Heart	1.0	Kidney	1.0

We were interested in knowing if all the amino acids participate in this reaction and, if so, to what extent. Accordingly, the activity of the individual amino acids to carry out the PP-ATP exchange was tested with an *E. coli* extract (table 4). Only 8 amino acids are active in catalyzing the exchange reaction. The complete mixture of the 19 amino acids is far more active than any individual amino acid, but the full activity of the complete mixture could be obtained by the combination of the 8 amino acids. These data suggest that more than one enzyme is involved in activating these 8 amino acids. Other evidence based on fractionation procedures also indicates the presence of several enzymes. Thus Berg ('56b) isolated from yeast an enzyme that is specific for the activation of methionine, Davie *et al.* ('56) isolated a tryptophan-specific enzyme from beef pancreas, and Schweet ('57) obtained a tyrosine-specific enzyme from pig pancreas.

TABLE 4

Catalysis of PP^{32}-ATP exchange by individual amino acids in Escherichia coli[a]

AMINO ACID	RATE OF PP^{32} EXCHANGE	AMINO ACID	RATE OF PP^{32} EXCHANGE
	µmoles/hr/ml of E. coli extract		*µmoles/hr/ml of E. coli extract*
Alanine	0	Serine	0
Arginine	0	Threonine	0
Aspartic acid	0	Tryptophan	20
Asparagine	0	Histidine	30
Glutamic acid	0	Phenylalanine	48
Glutamine	0	Methionine	122
Glycine	0	Tyrosine	146
Hydroxyproline	0	Valine	146
Lysine	0	Leucine	168
Proline	0	Isoleucine	248
		Complete mixture	416

[a] From DeMoss and Novelli ('56).

We were somewhat surprised to find that only 8 amino acids are active in the microbial PP-ATP exchange system. This result could be a real phenomenon, or it could have been caused by the inactivation of certain enzymes during the preparation of the extracts. We therefore prepared extracts from several other rather different physiological types of microorganisms and compared the activity of individual amino acids (table 5).

Since the same qualitative result is obtained with extracts of organisms of such greatly different nature, it seems unlikely that the amino acid specificity noted is an experimental artifact resulting from enzyme destruction. The activity of individual amino acids in a crude extract prepared from rat liver was examined, and here again (table 6) only the same

TABLE 5

Catalysis of PP^{32}-ATP exchange by individual amino acids in several microbial extracts

AMINO ACID	RATE OF PP^{32} EXCHANGE			
	E. coli	*A. aerogenes*	*M. lactilyticus*	*N. crassa*
	µmoles/hr/mg protein			
Alanine	0	0.33	0	0.14
Arginine	0	0	0	0
Aspartic acid	0	0	0	0.14
Asparagine	0	0	0	0.14
Glutamic acid	0.07	0	0	0.14
Glutamine	0	0	0	0
Glycine	0	0	0	0
Hydroxyproline	0	0	0.08	0
Lysine	0	0	0	0
Proline	0	0	0	0
Serine	0	0	0.41	0.36
Threonine	0	0	0	0.14
Tryptophan	0.36	0	0.98	0.14
Histidine	0.57	0	0	0.44
Phenylalanine	0.95	0.33	0	0.55
Methionine	2.53	2.02	0.34	0
Tyrosine	3.00	2.37	0.41	0.87
Valine	3.00	9.14	2.07	0.65
Leucine	3.46	10.04	3.34	1.60
Isoleucine	5.12	16.59	5.01	0.16
Cysteine	. . .	4.73	1.59	0.55
Complete mixture	7.30	21.00	6.40	1.67

TABLE 6

Catalysis of PP^{32}-ATP exchange by rat liver enzymes as a function of individual amino acids

AMINO ACID	RATE OF PP^{32} EXCHANGE	AMINO ACID	RATE OF PP^{32} EXCHANGE
	µmoles/hr/ml		*µmoles/hr/ml*
None	1.29	Phenylalanine	1.89
Glutamic acid	0.94	Histidine	1.89
Serine	1.01	Tryptophan	2.27
Arginine	1.03	Valine	2.99
Alanine	1.12	Isoleucine	3.75
Lysine	1.20	Cysteine	4.44
Hydroxyproline	1.22	Methionine	4.48
Glycine	1.25	Tyrosine	4.56
Proline	1.31	Leucine	5.02
Threonine	1.43	Complete mixture	10.82

amino acids are active. We have no ready explanation for this series of observations, but we do believe that these amino acids may represent a group of acids that are primarily activated, and that the remaining inactive ones are activated through a transacylation mechanism not involving a PP exchange.

During these studies, none of the components seemed to disappear during the reaction. We therefore conducted a balance study of the reaction to see if any net change could be observed (table 7).

TABLE 7

Balance study of PP^{32}-ATP exchange reaction in Escherichia coli[a]

	INITIAL		FINAL		EXCHANGE
	Concn.	Specific activity	Concn.	Specific activity	
	mM	*cts/min/μmole*	*mM*	*cts/min/μmole*	*per cent*
ATP	10.8	0	10.5	410	56
PP^{32}	11.5	1390	11.7	1095	
Amino acid mixture	10.0	....	10.3	....	

[a] From DeMoss and Novelli ('56).

Concentrations of ATP, PP, and amino acids measured after 15 minutes incubation showed that all reactants were present in their initial concentration in spite of the occurrence of 56% exchange of isotope. This observation suggests that ATP reacts with the amino acid, splitting off PP in a rapidly reversible manner without the accumulation of significant amounts of intermediate products. This result could be explained by assuming that the intermediate remains tightly enzyme bound, with little or no tendency to dissociate during the reaction.

Hoagland has shown, and we have confirmed, that ATP is broken down in the presence of high concentrations of hydroxylamine; and adenosinemonophosphate (AMP), PP, and the corresponding aminoacyl hydroxamate are produced in stoichiometric amounts (Hoagland, '55). This suggests that the amino acids are activated through their carboxyl group.

It is interesting that the rate of exchange is invariably several times as fast as the rate of hydroxamate formation, whether tested with a complete amino acid mixture or with leucine as substrate (table 8). This may reflect a low order of reactivity of the intermediate with hydroxylamine when the former is enzyme bound. Using hydroxylamine to trap the activated amino acids, a number of workers have reported the formation of hydroxamates of some of the amino acids that apparently do not catalyze the PP-ATP exchange reaction. In comparing the rate of hydroxamate formation and PP-ATP exchange catalyzed by individual amino acids, we observed hydroxamate formation from amino acids that do not stimulate the PP-ATP exchange (table 9); but more im-

TABLE 8[a]

Comparison of rate of exchange reaction with the formation of amino acid hydroxamate

SUBSTRATE	HYDROXAMATE	EXCHANGE	$\frac{\text{EXCHANGE}}{\text{HYDROXAMATE}}$
	μmoles/hr/ml of E. coli extract		
Leucine	6.0	168	28
Amino acid mixture	8.7	416	48

[a] From DeMoss and Novelli ('56).

TABLE 9

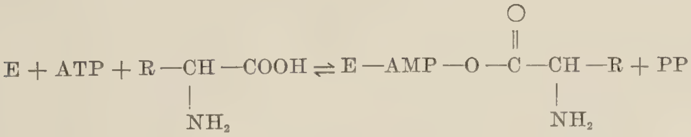
Comparison of rate of exchange with formation of hydroxamate as a function of individual amino acids in Escherichia coli

AMINO ACID	HYDROXAMATE	EXCHANGE	AMINO ACID	HYDROXAMATE	EXCHANGE
	μmoles/ml of E. coli extract/hr			*μmoles/ml of E. coli extract/hr*	
Alanine	0.40	0	Serine	0.55	0
Arginine	0.95	0	Threonine	0.65	0
Asparagine	16.60	0	Tryptophan	1.25	20.5
Aspartic acid	22.35	0	Histidine	0.10	31.0
Glycine	0.40	0	Phenylalanine	1.50	49.0
Glutamic acid	7.75	0	Methionine	1.05	125.0
Glutamine	10.35	0	Tyrosine	3.35	149.0
Hydroxyproline	0.20	0	Valine	0	149.0
Lysine	0.0	0	Leucine	0.15	172.0
Proline	0.15	0	Isoleucine	0.15	254.0

portant, with the amino acids that do participate in the exchange reaction, there was no correspondence between the magnitude of the exchange and hydroxamic acid formation. This suggests that the PP exchange is a measure of the primary activation step and that perhaps hydroxamic acid formation is dependent on the chemical nature of the intermediate or that it is a measure of a subsequent event, such as transacylation or transport.

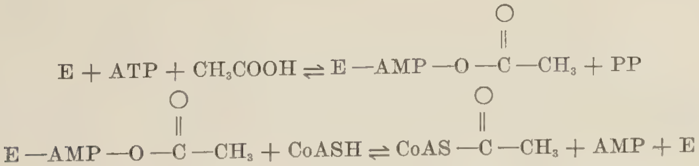
Up to this point then we know that: (1) The enzyme system catalyzes a rapid exchange of PP with ATP that is dependent on amino acids, but in which no net reaction can be seen. This suggests that an enzyme-bound intermediate may occur during the reaction. (2) The enzyme system does not catalyze an exchange between AMP and ATP, either in the presence or absence of amino acids (Hoagland, '55). (3) In the presence of high concentrations of hydroxylamine, AMP, PP, and the corresponding hydroxamate of the amino acid are formed in stoichiometric amounts. This indicates that during the PP-ATP exchange the carboxyl group of the amino acid becomes activated.

The most reasonable interpretation of these findings is that the intermediate is an anhydride between the phosphate group of AMP and the carboxyl group of the amino acid, as shown in equation (3).



$$\mathrm{E} + \mathrm{ATP} + \mathrm{R}-\underset{\mathrm{NH_2}}{\underset{|}{\mathrm{CH}}}-\mathrm{COOH} \rightleftharpoons \mathrm{E}-\mathrm{AMP}-\mathrm{O}-\overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}}-\underset{\mathrm{NH_2}}{\underset{|}{\mathrm{CH}}}-\mathrm{R} + \mathrm{PP} \qquad (3)$$

This is analogous to the formulation by Berg of acetyl-AMP as an intermediate in the formation of acetyl-CoA, as shown in equation (4).



$$\mathrm{E} + \mathrm{ATP} + \mathrm{CH_3COOH} \rightleftharpoons \mathrm{E}-\mathrm{AMP}-\mathrm{O}-\overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}}-\mathrm{CH_3} + \mathrm{PP}$$

$$\mathrm{E}-\mathrm{AMP}-\mathrm{O}-\overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}}-\mathrm{CH_3} + \mathrm{CoASH} \rightleftharpoons \mathrm{CoAS}-\overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}}-\mathrm{CH_3} + \mathrm{AMP} + \mathrm{E} \qquad (4)$$

Berg synthesized acetyl-AMP chemically and showed that it was rapidly converted to ATP in the presence of PP or to acetyl-CoA in the presence of CoA (Berg, '56a, c). By analogy to this system, we proposed aminoacyl-AMP as the intermediate in the amino acid activation system. Accordingly, DeMoss *et al.,* ('56, a, b) synthesized leucyl-AMP by treating the disilver salt of AMP with the acid chloride of leucine, and testing for activity in a partially purified system.

When leucyl-AMP is incubated with the purified enzyme and inorganic pyrophosphate, it is rapidly converted to ATP (table 10). The data also demonstrate that the conversion is dependent on both the enzyme and inorganic pyrophosphate. These results are consistent with the idea that the amino acids are activated by reaction with ATP to form an aminoacyl-AMP compound with the elimination of inorganic pyrophosphate in a freely reversible reaction. The results of the experiment of table 8, in which a net reaction cannot be demonstrated, suggest that the aminoacyl-AMP intermediate is tightly enzyme bound and has little or no tendency to dissociate. When the enzyme is incubated with C^{14}-leucine, ATP, and an excess of leucyl-AMP, none of the isotope can be recovered as leucyl-AMP. This lends further support to our assumption, and suggests that any enzymically formed leucyl-AMP did not equilibrate with the added leucyl-AMP.

TABLE 10

Rate of formation of ATP from leucyl-AMP

TIME	COMPLETE SYSTEM		NO ENZYME		NO PP^{32}	
	LEUCYL-AMP Δ μmoles	ATP Δ μmoles	LEUCYL-AMP Δ μmoles	ATP Δ μmoles	LEUCYL-AMP Δ μmoles	ATP Δ μmoles
min						
1	−4.8	+4.3	−0.1	+0.2	0.0	0.0
3	−9.0	+6.1	−3.0	+0.1	−2.8	0.0
5	−9.5	...	−5.7	...	−5.9	...

Conditions: 300 μmoles of phosphate buffer, *p*H 6.8; 90 μmoles of $MgSO_4$; 30 μmoles of PP^{32}; 13.5 μmoles of leucyl-AMP; 0.8 ml of purified *E. coli* extract in a final volume of 3.0 ml. Incubation was at 37°C.

This situation is very reminiscent of the first stages in the activation of acetate as described by Berg ('56a). "Active acetate," in its transport form as acetyl-CoA, is known to undergo a series of condensations to form the complex polymer, cholesterol. Cholesterol formation can be broken up into a three-stage process that may have a superficial similarity to protein synthesis (fig. 2). A consideration of cholesterol synthesis, may therefore give some clues to a mechanism for protein synthesis.

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1. Activation of acetate
   $\mathrm{E} + \mathrm{ATP} + \mathrm{Ac} \rightleftharpoons \mathrm{E}—\mathrm{AMP}—\mathrm{Ac} + \mathrm{PP}$
2. Transport of activated acetate
   $\mathrm{E}—\mathrm{AMP}—\mathrm{Ac} + \mathrm{CoASH} \rightleftharpoons \mathrm{CoAS}—\mathrm{Ac} + \mathrm{E} + \mathrm{AMP}$
3. Polymerization of acetate
   $\mathrm{n}\ (\mathrm{CoAS}—\mathrm{Ac}) \rightarrow$ cholesterol

---

Fig. 2. Three-stage synthesis of cholesterol.

Figure 2 shows that the first step in cholesterol synthesis requires the activation of the carboxyl group of acetate by reaction with ATP, which leads to the formation of an enzyme-bound acyl-AMP complex and eliminates inorganic pyrophosphate.

Step 2 describes the removal of the activated acetate by reaction with CoA, and results in the formation of acetyl-CoA. In this process, the activated acetate is converted from a mixed anhydride (acetyl-AMP) to a thiol ester (acetyl-CoA), with a drop in free energy (Jenks, '57). Yet the thiol ester still has enough energy to undergo the polymerization described in stage 3. Significantly, acetyl-CoA is the first product in the activation of acetate that accumulates as a free intermediate. Since acetyl-CoA undergoes many reactions in addition to cholesterol synthesis, it may properly be considered as the transport form of "active acetate."

By analogy, we can formulate protein synthesis as a three-stage process (fig. 3).

1. Activation of amino acids
   $E + ATP + AA \rightleftharpoons E—AMP—AA + PP$
2. Transport of activated amino acids
   $E—AMP—AA + X \rightleftharpoons X—AA + E + AMP$
3. Polymerization of activated amino acids
   $n\ (X—AA) \rightarrow \text{protein}$

Fig. 3. Three-stage synthesis of protein.

Reaction (1), the activation of the amino acids, is the process we have been discussing here in detail; it has many properties in common with the activation of acetate. Since it seems necessary that the activated amino acids be removed from the enzyme surface and transported to regions in the cell where protein synthesis takes place, a transport stage similar to acetyl-CoA is suggested. The hypothetical acceptor of the activated amino acids is designated "X," since its existence and chemical nature are not known. Holley ('57) and Hoagland and Zamecnik ('57) have, however, produced evidence suggesting that a soluble form of RNA may be the acceptor of the activated amino acids.

The third stage represents the condensation of the activated amino acids into the long polypeptide polymer that is protein. This step in protein synthesis is vastly more complex than the corresponding step in cholesterol synthesis, in which the various 2-carbon units that make up the polymer have no special identity and apparently are used in a random fashion. Also in cholesterol synthesis, intermediates more complex than acetate and less complex than cholesterol occur. In protein synthesis, on the other hand, present information indicates that proteins are constructed from free amino acids without the accumulation of free polypeptide intermediates (Spiegelman *et al.*, '55). The complexity of this third stage arises from the necessity for the activated amino acids to be aligned in a predetermined order before condensation. Polymerization and perhaps a specific type of folding must follow. As mentioned, the mechanism of this complex process has invited considerable speculation, especially in attempts to describe the chemical nature of the template and how it might act.

It would be interesting, therefore, to examine some of the parameters that might be involved in protein synthesis and to erect some restrictions on our speculations.

We have already mentioned the high degree of functional and structural specificity exhibited by proteins, and that is passed on from generation to generation. Thus it is clear that a certain amount of information is necessary to construct a unique protein; how much or how little is not yet known. For the sake of this discussion, we will assume that each and every peptide bond in a protein molecule must be exactly specified. This is the most complex situation. Should later data indicate that somewhat less information is required, a simplification can be made.

Since the information regulating protein synthesis must be passed to succeeding generations, it is clear that the *prime* source of information is in the genes. The site of this information could be in the protein of the chromosome, in the DNA, or in the deoxyribonucleoprotein. We can then inquire with Spiegelman ('57), "is DNA personally involved in protein synthesis, i.e., is it the template?"

Experimental information from various sources indicates that protein synthesis can continue in the absence of nuclear DNA (Vanderhaeghe, '52; Mazia and Prescott, '55). Thus in enucleated amoeba and Acetabularia, protein synthesis can continue for weeks in the apparent absence of DNA. Physical agents such as ultraviolet light and nitrogen mustard, which destroy DNA, do not affect protein synthesis (Pardee, '54). It is therefore evident that DNA does not participate directly and immediately in all protein synthesis.

The specific information regulating protein synthesis, and that is carried into a cell via genetic material, must therefore be transmitted to the cytoplasm. The most likely possibility is that an organizer or template specific for each protein is synthesized by the nucleus and excreted into the cytoplasm, where it can act catalytically at least for a while in the absence of DNA.

The necessary characteristics of the organizer system are: (1) it must contain enough information to regulate the sequence of amino acids in the protein whose synthesis it determines; (2) it must be capable of acting catalytically; and (3) it must be synthesized in the nucleus under the influence of DNA. Since the data from the literature discussed up to this point suggest that DNA is not directly and immediately involved in protein synthesis, the remaining macromolecules that should be seriously considered as candidates for the organizer system are RNA and protein itself.

Because of the original observations of Brachet ('47) and of Caspersson *et al.* (41- '44) that tissues in a high state of protein synthesis also have a high content of RNA, it is rather generally believed that RNA is somehow implicated in protein synthesis. These experiments have been confirmed and extended and still the conviction remains that in some manner RNA and protein synthesis are related.

A number of people have developed theories in which they postulated that RNA is the specific template material (Koningsberger and Overbeek, '53; Dounce, '51-'52; Borsook, '56). The theories expressed by these investigators differ somewhat in specific details, but in general they all specify that a specific RNA acts as a framework on which the amino acids are lined up in a proper sequence governed by the information contained in the RNA. These theories further specify that the amino acids are bonded to the phosphate groups in RNA. Borsook and Koningsberger and Overbeek visualized the carboxyl group as being attached to phosphate; Dounce assumes that the amino group is attached.

Let us examine some of the experimental evidence concerning RNA and protein synthesis with a view toward deciding whether RNA could act as the specific organizer.

Is RNA being synthesized in the nucleus? There is considerable evidence, obtained from feeding various isotopic precursors of RNA to organisms and tissues, indicating that the specific activity of newly formed RNA is greater in the nucleus than in the cytoplasm (Allfrey and Mirsky, '55; Goldstein and Plaut, '55). On the other hand, Brachet and Szafarz ('53) showed that the enucleated half of Acetabularia is capable of incorporating C^{14}-labeled orotic acid, but at a rate much lower than that of the nucleated half. This experiment suggests that at least some synthesis or turnover of RNA can occur in the absence of nuclear DNA.

The experiments of Prescott ('57) with nucleated and enucleated amoeba suggest that RNA is synthesized *exclusively* in the nucleus. Using C^{14}-uracil and following the course of labeling with the radioautographic technique, he found that the labeling first appeared and rose to a peak in the nucleus and later appeared in the cytoplasm. Furthermore, enucleation of the amoeba stopped completely the incorporation of C^{14}-uracil into the cytoplasmic RNA, even though the amoeba could still take up the uracil from the medium and convert it to acid-soluble nucleotides.

It is difficult to generalize about the role of the nucleus in RNA synthesis in view of the apparent contradictory evidence from these two laboratories. It should be mentioned that Acetabularia is a photosynthetic organism capable of much more independent metabolism than amoeba. In Acetabularia, although the evidence does not support the idea that RNA synthesis is exclusively under nuclear control, it is evident that the nucleus has at least a marked influence on the synthesis of RNA.

Can RNA carry specific information? Since four different bases occur in RNA, it could carry enough information to regulate protein synthesis if certain restrictions are imposed. The question of information or coding is rather complex and unclear at the moment, so we will not dwell upon it here. Suffice it to say that, if each amino acid in the polypeptide sequence must be exactly specified, then RNA cannot do it if we assume one base for each amino acid. If, however, according to Gamow ('55), three bases or a triad are used to specify one amino acid, then RNA could easily carry sufficient information to specify a unique protein. The assumption of a triad as the basic coding unit, however, encounters structural problems, as we shall see in a moment.

Can or does RNA act catalytically? Using an RNA model as a template and assuming a helical structure such as the Watson and Crick model for DNA, we find that the bases are separated from each other by 3.4 A along the fiber axis and the phosphate groups are 7.0 A apart (Watson and Crick, '53; Schwartz, '55).

If the activated amino acids are to be positioned on the phosphate groups, they would be too far apart for spontaneous condensation into peptide bonds. This applies if only one base specifies a single amino acid; and we have already shown that such a circumstance does not provide sufficient information to describe a unique protein. If we use Gamow's notion of a triad as the coding unit, the situation becomes even worse, since the activated amino acids would now be even farther separated.

To make a peptide bond, the carboxyl and amino groups must be brought closer together than the exclusion diameter (2.28 A), hence the use of a triad to specify the amino acid and the phosphate groups as recipients would never permit condensation to occur. This circumstance speaks against RNA, *by itself,* being the organizer. And now we have our first restriction on the nature of the template. It must be constructed so that the carboxyl carbon of one amino acid is brought as close as 2.0 A to the amino nitrogen of an adjacent amino acid. This restriction, however, does not exclude RNA from playing a role in the organizer system, and we should examine further evidence of its participation.

There is some evidence that RNA acts catalytically in protein synthesis. For example, the over-all composition of *E. coli* indicates that there are fifteen amino acid residues in the protein of *E. coli* for each base in the RNA (Pardee and Prestidge, '56). Since other evidence indicated that in *E. coli* RNA does not turn over (Manson, '53; Siminovitch and Graham, '56), the amino acid-RNA ratio of 15:1, suggests that if RNA acts as template material it does so either catalytically or via a mechanism involving less than one base to one amino acid.

There is still other evidence suggesting that RNA does not act catalytically. In experiments with a uracil-requiring strain of *E. coli,* Pardee and Prestidge ('56) showed that RNA and protein synthesis proceed together until the limiting quantity of uracil is exhausted, at which time both RNA and protein synthesis cease. If uracil is now added back, both processes are resumed until the uracil once again is exhausted and both processes cease again. This observation suggests that protein synthesis requires simultaneous RNA synthesis. A similar conclusion was reached by Spiegelman *et al.* ('55) and by Creaser ('55), using quite different systems. It should be mentioned that most of the evidence for the direct involvement of RNA in protein synthesis has come from experiments of adaptive enzyme formation in bacteria, and it is possible that adaptive enzyme formation is a special case of protein synthesis with particular requirements that cannot be generalized to include all protein synthesis. Nevertheless, the available information suggests rather strongly that RNA acting alone cannot be the organizer system.

Protein as template material. The only other macromolecule remaining that could contain enough information to act as a template is protein itself; and certainly proteins in the form of antigens can induce the synthesis of new proteins. The protein itself should therefore be examined for properties necessary for it to act as the organizer system.

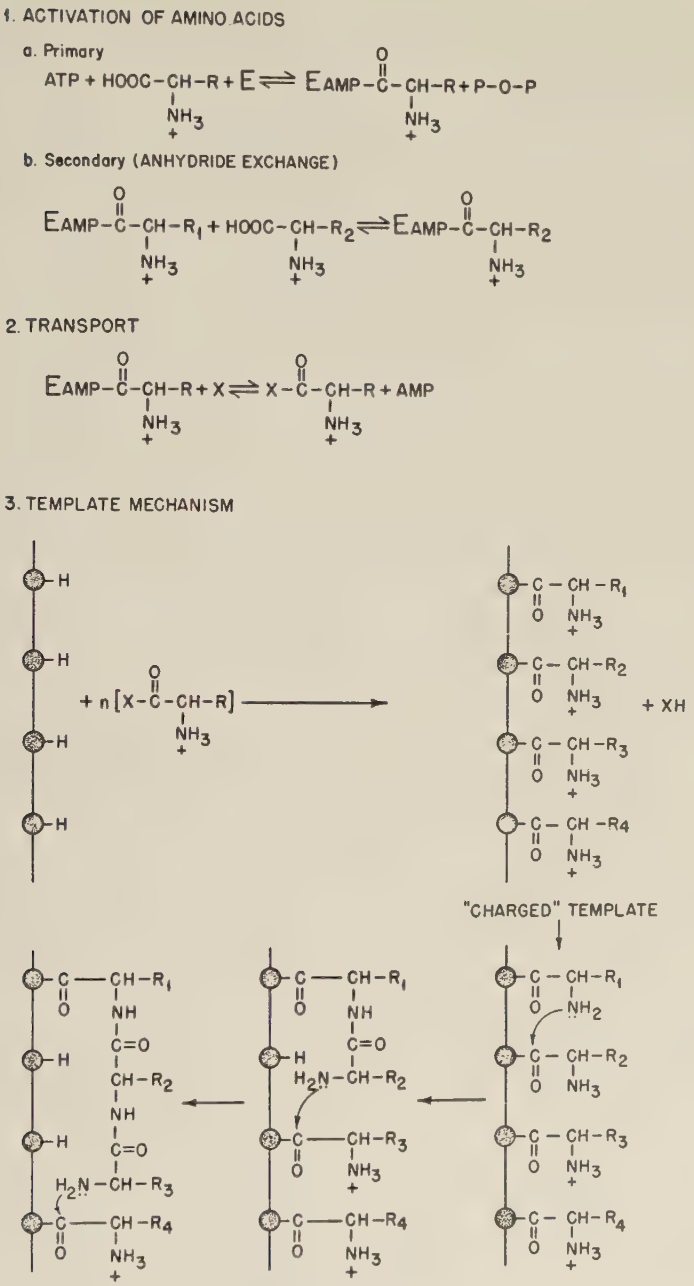
One such property is that the organizer system must be synthesized by the nucleus under the influence of DNA. Allfrey and his coworkers ('57) showed that protein is indeed synthesized in the nucleus, and obtained some circumstantial evidence that DNA may be involved in this synthesis. The other property is that the template must be able to carry information. Certainly, protein has sufficient information to carry a code for the formation of new protein.

Can protein act catalytically? There is no evidence for a direct role of protein in protein synthesis, and such evidence would be difficult to obtain. The closest approach to this point is antibody formation induced by a protein antigen. But since polysaccharide can also bring about the formation of antibody, it would seem that such a synthesis does not require the exclusive participation of protein.

Although a protein has potential binding sites for the alignment of activated amino acids that are close enough to allow spontaneous condensation of the amino acid residues, there is no simple chemical model available upon which to construct a mechanism by which protein can function as a template. It seems most probable that the template is a linear structure, so that the amino acids can be lined up in sequence. It is difficult, however, to visualize the unfolding of an already folded protein or the maintenance of a protein in an unfolded state to provide a specific surface upon which the amino acids could be aligned.

These considerations, then, make it unlikely that protein alone can function as a template. More likely, RNA, together with protein in the form of ribonucleoprotein, acts as a functional organizer to provide information governing the sequence of amino acids as well as providing a structure on which the spontaneous polymerization can take place. Such a structure would have all the desirable properties that have just been enumerated for both RNA and protein; namely, (1) both are synthesized in the nucleus; (2) together they could carry more than enough information for the synthesis of new protein; (3) RNA could act to keep the protein part of the structure in an extended linear arrangement; and (4) either or both partners could provide the catalytic property of the template. Clearly, then, there seems to be a lack of conclusive experimental evidence to permit a definite decision about the chemical nature of the template or organizer system.

We can then summarize this rather general picture of protein synthesis in figure 4.


1. ACTIVATION OF AMINO ACIDS
a. Primary
ATP + HOOC–CH–R + E ⇌ EAMP–C(=O)–CH–R + P–O–P
NH3 +
b. Secondary (ANHYDRIDE EXCHANGE)
EAMP–C(=O)–CH–R1 + HOOC–CH–R2 ⇌ EAMP–C(=O)–CH–R2
NH3 +
2. TRANSPORT
EAMP–C(=O)–CH–R + X ⇌ X–C(=O)–CH–R + AMP
NH3 +
3. TEMPLATE MECHANISM
H
+ n [X–C(=O)–CH–R]
+ XH
C – CH – R1
C – CH – R2
C – CH – R3
C – CH – R4
O
NH3
NH2
NH
C=O
H2N–CH–R2
H2N–CH–R3
"CHARGED" TEMPLATE

CONDENSATION REACTIONS

Figure 4

Step 1 is the reaction already discussed at some length; namely, the activation of the amino acids.

The transacylation step is introduced to provide a mechanism for activation of those amino acids that do not participate directly in step 1. Such a reaction could explain the formation of hydroxamates by those amino acids that do not catalyze the PP-ATP exchange (table 9).

Since the activation reactions take place in the soluble portions of the cell, step 2 is introduced to allow for transport of the activated amino acids to the site or sites in the cell where protein synthesis takes place. As mentioned, Holley ('57) and Hoagland and Zamecnik ('57) obtained evidence suggesting that "X" may in fact be some form of RNA itself.

Step 3 is a schematic representation of one way in which a template may act. This is highly speculative and much will depend on the chemical nature of the template. In any case, certain provisions must be fulfilled regardless of the chemical nature of the template. Some of these provisions are: (1) The template must be able to select specific amino acids to be arranged in a predetermined sequence. (2) The amino acids must be lined up in such a manner that the carboxyl group of one amino acid can be brought to within 2.2 A of the amino group of an adjacent amino acid. (3) The activated amino acids must be prevented from spontaneous condensation until the template is fully charged or; alternatively, the polymerization must be controlled in such a manner that it can proceed in only one direction along the template (Dalgliesh, '57).

We have attempted to provide here a biochemical background of the general principles involved in protein synthesis, as a framework upon which the synthesis of antibodies can be projected.

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CONCEPTS OF PROTEIN SYNTHESIS IN RELATION TO ANTIBODY FORMATION

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THREE FIGURES

INTRODUCTION

Studies of protein synthesis in the last few years have increased our knowledge of this process to the point at which at least a general outline is perceptible. This is not true for antibody synthesis, largely because it represents a very special, probably more complex, type of protein synthesis. Furthermore, the process has not long been amenable to study by certain powerful biochemical techniques, since it has been an *in vivo* system only. Antibody formation is relevant to the general problem of protein synthesis because it is the synthesis of new protein. Convincing proof that new protein is made during antibody formation and that the existing γ-globulin is not converted to Ab has been presented by Green and Anker ('54) and Gros *et al.* ('52).

This paper will be an attempt to review the general principles of protein synthesis and to treat the synthesis of circulating antibody as a special example of the general process.

PROTEIN SYNTHESIS

The nature of antibody

Since this discussion will be confined to circulating antibody (Ab), its relation to "normal" γ-globulin is pertinent.

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Physical methods have shown γ-globulin to be heterogeneous (Smith and Jager, '52). In the rabbit, where the normal γ-globulin has a unique N-terminal pentapeptide, Ab to various antigens (Ag) all had the same terminal pentapeptide. In species where normal γ-globulin contained more than one N-terminal group, specific Ab to different pneumococcal Ag had the same varied complement of N-terminal groups as the original γ-globulin and as each other (McFadden and Smith, '55). This plus the immunological facts that (1) there is wide variation in the type of Ab produced under different conditions by a single Ag, and (2) the reacting sites are probably a small area of the molecule, make it seem logical that many kinds of protein might serve as Ab. The biological requirement is for the proper cell type or types, which can respond to an Ag by producing a protein with a specific configuration on a small part of its surface.

The two factors that might affect the configuration at the combining site are the amino acid sequence and the "folding." The difference between normal and sickle-cell hemoglobin has been reported to be a change in a single amino acid in a particular peptide (Ingram, '56). On the other hand, the amino acid sequence within a particular locus could be the same, but a different configuration might result from a different folding of the molecule (Pauling, '40). It is also probable that a locus with the same specific configuration could, in different proteins, have different amino acid sequences. This might be the case in different proteins with similar Ab action. An example of this is insulin, in which the amino acid sequence in various species differs, but all show the same kind of biological activity.

Most proteins, as well as Ab, have a specific three-dimensional structure, or "folding." Part of this "folding" is caused by cross-linkages, which are formed specifically as an intrinsic part of the synthesis of the molecule. Haurowitz ('50) presented a similar concept. One example of this is the intramolecular –S–S– bond in insulin, which cannot be formed spontaneously. Ribonuclease contains specific –S–S– bonds and specific bonds from tyrosine-hydroxyl to carboxyl groups. Phosphate ester linkages and even hydrogen bonds may also be formed specifically.

It is not known how the specific bonds that regulate the final three-dimensional structure of proteins are produced. The formation of the specific configuration, however, must take place very near in time and space to the formation of the amino acid sequence, and it seems logical to assign the formation of both to the same genetically controlled process. On this basis, the formation of a specific Ab site, whether caused by a change in amino acid sequence or "folding," is attributed to a change in a template involved in protein synthesis.

Templates in protein synthesis

It is generally agreed that some form of template mechanism is involved in protein synthesis, rather than a synthesis via free peptide intermediates (Borsook, '56; Steinberg *et al.*, '56). The evidence that deoxyribonucleic acid (DNA) is not the template material has been summarized by Novelli and DeMoss in the preceding paper. An exception to this may be protein synthesis in the nucleus (Allfrey *et al.*, '57). On the other hand, some association of ribonucleic acid (RNA) with protein synthesis is well established and RNA has been implicated as template material (see Novelli and DeMoss for references). The following data indicate that templates for protein synthesis probably consist of an RNA-protein complex (RNP). The RNA in microsomes, where most of the protein synthesis of the cell occurs, is not free, but bound to protein. If microsomes are fractionated with deoxycholate, a protein and an RNP fraction can be separated. The RNP fraction becomes labeled with a C^{14}-amino acid faster than the protein fraction (Littlefield *et al.*, '55). The proteins of microsomes are heterogeneous physically and also incorporate C^{14}-amino acids at different rates (Simkin and Work, '57). The amino acid composition of protein extracted from RNP of the silk gland does not resemble that of silk fibroin, the main protein synthesized by these glands (Shimura *et al.*, '56). Thus there is a protein component of the microsomes bound to RNA that becomes labeled, but probably is not the direct precursor of the protein being synthesized, although parts of the protein of the RNP might be incorporated into product protein. The RNP of microsomes comes closest to our concept of a template for protein synthesis.

The problem of whether the template acts catalytically or becomes nonfunctional after the synthesis of a single protein molecule was discussed by Gale ('56) and Spiegelman ('56). When RNA synthesis is blocked, adaptive enzyme synthesis ceases; however, some synthesis of constitutive protein may continue. The RNA of growing bacteria is quite stable (Hershey, '54) and the amount of protein synthesized is far in excess of the RNA. Thus a catalytic role for RNA is indicated. RNA synthesis is needed for hemoglobin synthesis in reticulocytes, but a catalytic action is indicated here also (H. Borsook and K. Shimura, unpublished data). It is probable that each template can synthesize a number of protein molecules before it becomes nonfunctional. Therefore, some RNA synthesis would always be needed for the production of constitutive proteins, but an absolute requirement for new RNA synthesis would obtain where a new protein is synthesized.

From a genetic point of view, we must consider that cells originally receive their "information" via the nucleus. Biochemical evidence concerning a mechanism for such "information transfer" comes from numerous observations that the components of the template, RNA and protein, can be synthesized in the nucleus (Brachet, '56; Allfrey *et al.*, '57). Enucleated cells, however, can synthesize some protein and RNA, the quantity depending on which organism is studied, but are eventually exhausted. In bacteria, thymineless mutants that cannot synthesize DNA can continue making RNA and protein (Cohen, '57). Furthermore, treatments that might disrupt DNA do not impair protein synthesis in bacteria. Studies with cell-free bacterial preparations are less clear (Gale, '56; Spiegelman, '56). Cytoplasmic synthesis may, in general, be considered semiautonomous but ultimately dependent on the nucleus. A diagram of normal protein synthesis is shown in figure 1.

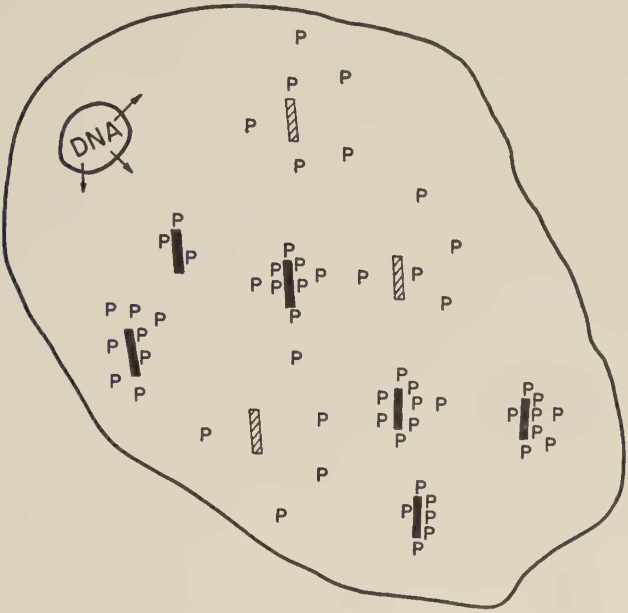

Fig. 1 Diagram of scheme for constitutive protein synthesis. The synthesis of a single protein species is shown. Information in some form, possibly ribonucleoprotein (RNP) itself, is shown as arrows coming from the nucleus. RNP template: solid bars are active; shaded bars are older, nonfunctional templates that have already produced their quota of protein (P).

Induced enzyme synthesis

The inducer in adaptive or induced enzyme synthesis is probably concerned with promoting a series of genetically determined events, and not with molding the enzyme configuration. Evidence for this idea is as follows: (1) A basal level of the enzyme is often present in the absence of inducer. (2) Ability to produce a particular inducible enzyme is a function of the genetic constitution, since certain strains are not inducible. Conversely, by mutation, enzyme formation in an inducible strain may become constitutive. (3) The presence of inducer can result in the formation of two ezymes; for example, penicillin induces the formation of two penicillinases, which appear to be different proteins (Pollock, '56). (4) The inducer may or may not be a substrate for the enzyme, and a substrate may or may not be an inducer (Monod, '56). (5) Other environmental conditions have a bearing on whether the enzyme is produced in the presence of inducer; e.g., the presence of glucose, which prevents the production of many inducible enzymes (Neidhardt and Magasanik, '56).

This evidence seems to limit the role of inducer to promoting a process already inherent in the cell strain being used. Lederberg ('56) presented evidence along similar lines.

The mechanism involved in the action of inducer is not known, although there is a great deal of information in this area. Mandelstam ('56) has reviewed the many theories in this field. Addition of inducer results in increased incorporation into RNA in resting cells but not in a net increase of the RNA (Chantrenne, '56). Inhibition of RNA synthesis prevents enzyme formation (Gale, '56; Spiegelman, '56). Furthermore, the early stages of induction (during the lag phase of enzyme production) are more sensitive to ultraviolet radiation than later stages (Halvorson and Jackson, '56). The action of inducer, then, seems to be related to the production of new RNA, specific for the synthesis of the induced enzyme. This RNA is considered to be a component of the RNP described previously.

According to this hypothesis, some templates are present in the absence of inducer and act to produce a low basal level of enzyme. A simplified representation of this is shown in figure 2. The arrows indicate that the flow of information is not impaired, but in contrast to figure 1, there are fewer templates and these are producing very little enzyme. The inducer probably stimulates the production of new templates in the cytoplasm as well as the production of enzyme by preexisting templates.

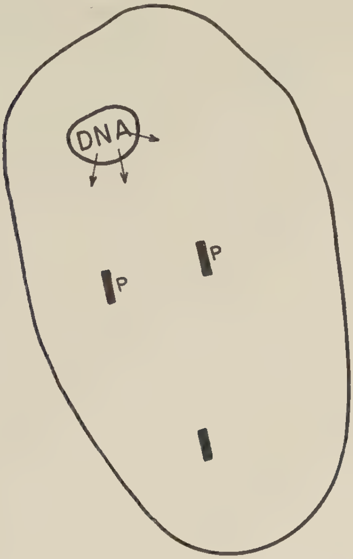

Fig. 2 Diagram of scheme for induced enzyme synthesis. The diagram shows the condition prior to the addition of inducer, which acts to produce the situation shown in figure 1. The solid bars are potentially active templates.

Antibody formation and induced enzyme synthesis

Antibody synthesis and induced enzyme formation may have much in common. There are, however, important differences between the two processes. The formulation of adaptive enzyme synthesis given indicated that (1) induced enzyme synthesis is possible only in cells having the genetic potentiality for making the particular enzyme, and (2) the inducer activates the machinery for enzyme production, but plays no part in molding the enzyme configuration.

Antibody formation differs from induced enzyme production in the following respects: (1) The cells that make Ab can form them in response to many Ag and can produce Ab proteins of many different specific configurations, depending on the Ag. The requirement is mainly for the Ag to be of large molecular weight and to be foreign. (2) The protein produced is structurally directed against the Ag that elicited its formation. (3) The degree of cellular reorientation involved in the two processes provides a marked contrast. As mentioned, resting cells can synthesize adaptive enzymes with no net increase in RNA (Chantrenne, '56). Antibody synthesis, however, is characterized by more-deep-seated changes involving cell division, differentiation, and growth (Coons, '56).

A biochemical model for antibody production

Since a new, specific configuration of a protein molecule is necessary for the formation of Ab and the stimulus for this is provided by the Ag, the role of Ag must be more complex than that of an inducer. The preceding discussion led to the concept that the amino acid sequence and configuration of proteins depended on the template and that the initial information needed for the formation of the template came from DNA. Therefore, a logical extension of the concept would indicate that the first action of Ag is on DNA. It is suggested that Ag then has a second function, analogous to that of an inducer. This seems to be the most direct hypothesis that can be derived from the biochemical data, and it receives support from immunological considerations in a later section of this paper.

The antigen modifies DNA. This could occur by combination of Ag with DNA, possibly at the locus of a gene concerned with globulin synthesis, or by some other mechanism. The action of Ag on DNA is considered to initiate the production of a modified DNA and a cell lineage that inherits the modified DNA. The DNA in these cells is capable of producing new templates specifically related to the Ag; only a small number of such templates may actually be produced.

The antigen functions as an inducer. According to this concept, Ag functions secondly as an inducer; i.e., it acts at the cytoplasmic level to stimulate the production of templates and of Ab by inactive templates. The mechanism here could involve an Ag-RNP combination or some more indirect action of Ag.

A representation of this scheme is shown in figure 3. Here the flow of information from DNA is lacking prior to the introduction of antigen. The formation of the DNA-Ag complex results in the production of a group of cells containing modified DNA. A few templates and some Ab are shown. All these cells are now considered to be in a condition similar to that shown in figure 2, i.e., before the introduction of inducer in the adaptive enzyme scheme. The inducer-like function of the Ag would convert these cells to full Ab production, analogous to the situation shown in figure 1. Although we have emphasized the two separate phases of Ag function, both would probably occur to some extent after a single injection of Ag because, after a latent period, the continuing presence of Ag would initiate the second phase, actual Ab production.

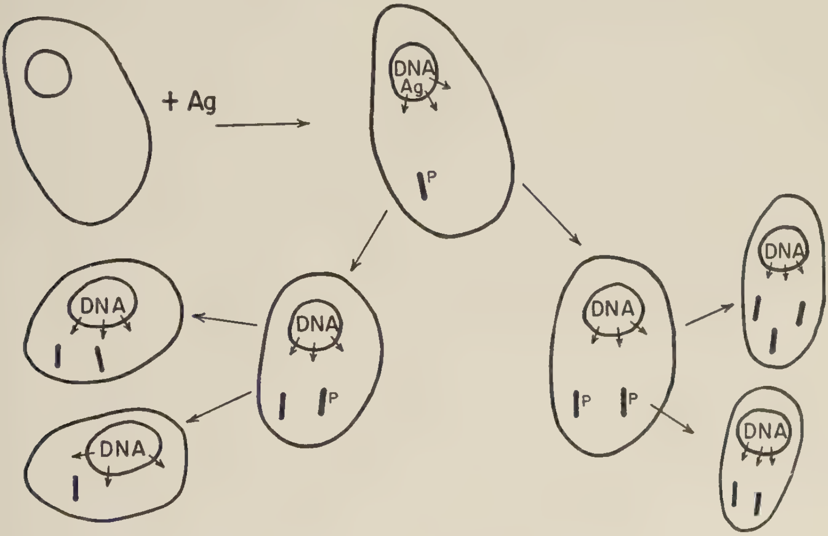

Fig. 3 Diagram of the scheme for the first action of antigen (Ag).

IMMUNOLOGICAL CONSIDERATIONS

The hypothesis derived from biochemistry will now be examined from an immunological point of view. It is appropriate here only to sketch briefly some of the relevant categories of information.

Morphology of antibody production. The most direct information probably derives from histological, cytological, and cytochemical studies of the uptake of Ag and of the subsequent behavior of cells involved in Ab formation. Dr. Coons' discussion of this subject, after this paper, renders elaboration of this evidence unnecessary here. Native protein Ag become associated with the nuclei of cells into which they penetrate. Such observations are, of course, consistent with the first phase of the hypothesis under consideration, in which Ag uptake would be followed by nuclear penetration and association with DNA, perhaps in a relatively small number of stem cells, to initiate a heritable change in these cells and to begin the production of new templates.

On present evidence, we may well doubt that Ag regularly penetrates the nucleus. In contrast to native proteins, modified ones, such as labeled bovine serum albumin, seem only rarely to be recognizable in cell nuclei, though nuclear penetration does occur (Coons, '56). Different Ag appear to behave differently in this respect. Such reservations need not be fatal to the hypothesis; in many instances, Ag breakdown products may be more directly involved than intact Ag (Garvey and Campbell, '56), and association of fragments of Ag with nuclear material might escape detection by the techniques applied, particularly if the number of cells initially and critically involved were small and the pertinent nuclear sites restricted in number. Observations that Ag may become associated with cytoplasmic granules (see Crampton and Haurowitz, '50) need not exclude nuclear effects; cytoplasmic associations, involved in early partial breakdown or later in connection with the second postulated action of Ag as an inducer, would be compatible with the hypothesis.

The behavior of pertinent cells after an initial antigenic stimulus, and in a secondary response, provides a stronger parallel with the hypothesis. The observed wave of mitotic mutiplication gives evidence of an early involvement of nuclear replication, and the distribution of Ab-producing cells in clusters (Coons *et al.*, '55; Leduc *et al.*, '55) suggests transmission of this potentiality through a line of cellular descent. Antibody first appears in "typical hematogenous stem cells" characterized by a large nucleus and basophilic cytoplasm. These cells multiply and differentiate, displaying a great increase in cytoplasmic RNA as judged by their staining reactions (Fagraeus, '48), by observations with ultraviolet light (Bing *et al.*, '45), and by measures of P^{32} uptake (Pinkus *et al.*, '54; see also Makinodan *et al.*, '54 for the sequence of changes in the chicken). The concentration of Ab increases in their cytoplasm, until colonies of mature plasma cells containing Ab are discernible.

This behavior represents a complete cellular reorientation with distinctive features not found in normal or induced protein synthesis. A new chain of events is initiated by Ag, characterized, in order, by a latent period, cell multiplication and differentiation via the formation of large amounts of cytoplasmic RNA, and Ab elaboration. This sequence supports the idea of changes at the nuclear level, initiated by the nuclear uptake of Ag, and followed by the production of new DNA, mitotic transmission to daughter cells, and the elaboration of new templates. The eventual second action of Ag as an inducer in the RNP template system is clearly conceivable on this basis.

Primary and secondary antibody responses. Upon first exposure to an Ag, a relatively few cells would begin to produce new templates, some of which would combine secondarily with available Ag and produce Ab. Antigen "available" in this sense, however, would be expected to decline over a relatively short interval, and the functional life of the template would be limited, as has been indicated for the general process of protein synthesis. At low levels of Ag, Ab production might cease not only for these reasons, but also because of dissociation of the Ag-template complex. The firmness of this combination might vary with the particular Ag, the stage of immunity, and other conditions. In adaptive enzyme formation, removal of inducer may result in various changes, ranging from complete cessation of enzyme production to the case (penicillinase, Pollock, '56) in which enzyme production continues unabated with less than 100 molecules of inducer bound per bacterial cell.

If the Ag, either through the use of adjuvants or for other reasons, were made available at an adequate but not excessive level over a longer period of time, a more persistent and higher Ab response would result. Proliferation of the original cells containing DNA-Ag would continue slowly after Ag was no longer available, with replication of the new DNA and slow production of inactive templates. The retention of "information," sometimes over long periods, would meet immunology's most difficult demands of biochemical models for Ab production.

Having once been placed in an inducible state, the system would be expected to react much more rapidly and vigorously to a second exposure to the Ag. This secondary response would be similar to the series of events postulated for the primary reponse but imposed upon a cell population containing inactive templates and conditioned, through derivation from cells that had contact with Ag originally, for rapid Ab elaboration. The ability of these conditioned cells to take up Ag more effectively might also play a part. There is a parallel for this possibility in the field of adaptive enzymes; Monod ('56) showed that inducer early brings into action a concentrating mechanism that causes a rapid uptake of inducer. According to the hypothesis, Ag at this stage of immediate Ab synthesis is functioning as an inducer, i.e., the specific pattern is already present in the RNP template. Also, this second function of Ag might be partially carried out by other relatively nonspecific compounds, just as induced enzyme formation may be initiated by materials other than substrates of the enzyme. Immunologists will note here an echo of a long-debated issue — the question of aspecific anamnestic responses (Burnet and Fenner, '49, p. 30; see Dixon and Maurer, '55, and Dubert, '56, for relevant data and discussions similar in important respects to the present consideration).

Changes commonly occur in the type and quality of Ab produced during immunization. These changes require explanation. In our hypothesis, the possible replication of templates in the absence of Ag may provide the required opportunity for change in the directions noted. It would doubtless be a mistake, however, to rely very heavily on this kind of explanation. The situation is obviously more complex than has been indicated in this brief outline; equilibrium situations, secondary effects and interactions, and perhaps a feedback mechanism may well be involved in this and in the phenomena next to be considered.

The disappearance of circulating Ag is often followed within a relatively short time by a cessation of Ab production (Dixon *et al.*, '56), but there are many reports that Ag persists in tissues for many weeks. It would seem that this Ag is not "available" to act as an inducer, although the first postulated function of Ag might continue during this period. It is not clear that the Ag that does persist is actually to be found in cells capable of elaborating Ab. Furthermore, Stark's evidence ('55) shows that persisting polysaccharide Ag actually declines in antigenicity, as judged by its effects when inoculated into normal mice. Antigen or antigenic fragments in the tissues might be blocked by combination with Ab or Ab-like material, and thus lose effective antigenicity. Such mechanisms may involve "afferent" blocks, which prevent access of the Ag to potentially Ab-forming elements, or "efferent," preventing the release of formed Ab, or a combination or succession of such effects. They become most conspicuous in immunological paralysis and "enhancement" (see review by Owen, '57). They may relate also to the refractory phases sometimes encountered during immunization, observed, for example, by Taliaferro and Taliaferro ('51) in connection with rabbit responses to sheep cells. Talmage, Freter, and Taliaferro ('56) cite evidence that Ab itself may suppress the formation of more Ab.

Suppressions of circulating Ab responses, achieved by injecting Ag into embryos or young birds and mammals, generally prove only partial and transient (e.g., Owen, '56; Simonsen, '56; Wolfe *et al.,* '57). There are, however, situations in which the injection of soluble Ag into newborn rabbits does induce a persistent and complete suppression of precipitin responses (e.g., Cinader and Dubert, '56). It should be noted that, in general, embryos and newborn animals do not produce globulins; cells that can later differentiate in the direction of Ab production appear late in development. Encounters, on the part of the individual, with foreign materials before the appearance of this system may well divert them into different channels. Some suppressions of immune responses achieved through injecting materials into embryos or young individuals may include direct effects on Ab formation or release, whereas others might relate to indirect effects, and be primarily concerned with a different system.

Such reservations apply even more strongly to the phenomenon of ''immunological tolerance,'' which involves the injection of viable homologous cells into embryos and the tolerance of individuals so treated to tissue transplants from the donor strain at maturity (Billingham *et al.,* '53). There is as yet no basis for ruling out, in this system, the possibility that the injected cells become established as persistent transplants in the treated individual. The nature of the transplantation Ag in cell-free preparations has been partially defined (Billingham *et al.,* '56). These Ag, though capable of inducing transplantation immunity in mature individuals, have so far not conferred tolerance when injected into embryos. Furthermore, this system depends on a cell-borne and cell-mediated type of immunity, in which no definitive cause-and-effect relation to serum Ab, of the sort considered in this paper, has yet been demonstrated (see Billingham and Brent, '56). Eventually these problems will need to be related to the subject under consideration; for the present, however, they must be set aside.

Effects of radiation. The basis for the apparent relative radioresistance of the secondary response in some systems and not in others may indeed be debated, but there seems to be little doubt that during Ab production in either response the system decreases in its vulnerability to radiation effects. This consideration of the effects of radiation on Ab production reflects the distinction by Dixon and his associates ('52) of a radiosensitive "adaptation phase" and a relatively radioresistant "production phase." Our hypothesis relates the early radiosensitive phase to the period of interaction between Ag and DNA, and perhaps to the formation of initial RNP. Consistently with other interpretations of radiation effects on biological systems, this treatment would incate that the preponderant effects involve DNA. The time relation between the injection of Ag and the effective period for radiation suppression, however, suggests that the critical effects of radiation are not immediate, but represent events accumulating over 6 or more hours, in particular species. It may be postulated that an effect of Ag under such circumstances is to convert an originally very vulnerable DNA site into a relatively stable DNA-Ag complex, and perhaps even to restabilize the DNA site in the early stages of radiation damage. In addition to the presence of Ag, such "repair" may well involve extracellular materials provided by other tissues. This postulate appears to be compatible with a variety of observations in the field, including the relation between Ag dose and radiation effects on Ab responses in both primary and secondary reactions (Talmage, Freter, and Thomson, '56). The proliferation of cells involved in normal Ab responses may also be related to radiation effects on DNA. Further directions that such considerations might take will be evident, but they are perhaps too speculative to justify elaboration here.

Nature of the postulated genetic change

There are, of course, ways in which cellular heredity may be modified without genetic change; for example, serotype transformation in ciliates (see Nanney, '57). Biochemical considerations, however, led to the suggestion that the simplest and most straightforward primary modification induced by Ag and leading to Ab formation would be a change in DNA. The hypothesis is that the DNA replicated from the DNA-antigen is a new DNA, having the configuration necessary for the production of Ab-forming templates. This change would not necessarily be a permanent one for the whole system, because the maturation of the primitive cells to Ab-producing ones involves a loss of ability to reproduce, as in red blood cells. To maintain such a population, in the absence of free Ag, either the new DNA or DNA-Ag could be maintained in stem cells that divide without maturation.

Another mechanism could involve transmission of Ag itself to the daughter cells, so that every cell had DNA-Ag. This might occur readily, because the original Ag-containing cell would be expected to contain many molecules of Ag. Alternatively, replication of the RNP templates might provide a basis for cytoplasmic transmisson. This last suggestion appears the least likely, and both biochemical and immunological evidence seem to point to Ag-modified DNA as a primary vehicle of transmission. More-sensational terminology would describe the postulated mechanism as one of directed somatic mutation, and most geneticists will experience an immediate antipathy to the careless enunciation of such a concept. On the other hand, many geneticists will recognize that in Ab production a unique modification of protein synthesis, that is, a change in specificity, must be involved; they may conclude that it is reasonable to turn to the seat of specific synthesis for such modification.

It should be emphasized that the postulated effects are on the properties of particular somatic cells, whose job on differentiation is to produce antibody. These cells, or their differentiated progeny, become factories for a particular kind of synthesis, just as other cells, for example, devote themselves to hemoglobin production. It is conceivable that in this particular environment a gene involved in globulin synthesis has a site within it subject to modification by foreign Ag or a fragment of Ag, such that the gene's own replication and the specific product for which it is responsible reflect that modification. Obviously, such effects must find expression only in the cells in which the synthetic sequence leading to Ab formation takes place. Other somatic cells, and the cells of the germ line, need not engage in such effects, and in fact must be insulated from them. Perhaps the pervasive machinery of this ''insulation'' may relate to the separate embryonic mechanism implied in the preceding discussion of immunological tolerance. In any case, a distinction between the metabolic treatment or ultimate effect of ''self'' as distinct from ''foreign'' components must be involved in the proper function of the integrated organism.

Theories of antibody formation

Earlier hypotheses suggesting the direct intervention of Ag in the determination of Ab specificity, either by affecting the amino acid sequence of the polypeptide chain (Breinl and Haurowitz, '30; Alexander, '32; Mudd, '32) or its ultimate folding in the formation of globulin molecules (Pauling, '40; cf. Haurowitz, '50, '53), have profoundly affected thought in the direction of template mechanisms. The germ of this concept is retained in our hypothesis, even to the extent of recognizing the possibility that Ag, or a fragment of Ag, may be retained in nuclear DNA. The direct action of Ag itself, serving as the template for the formation of Ab protein is, however, rejected, primarily because current biochemical evidence offers no basis for conferring protein specificities in this way, and instead suggests the intervention of an RNP template deriving its specificity from nuclear DNA. Furthermore, the persistence of the sensitive state (in contrast to the elaboration of Ab itself), and other considerations, offer difficulties for a hypothesis based on direct template action of Ag in Ab formation.

A hypothesis developed by Burnet and Fenner ('49) undertook to deal with some of these difficulties, but the identification of the entire process of Ab formation with adaptive enzyme formation, conceivable at the time, has since become untenable. Burnet ('56) developed a modification of the earlier theory, in which elements similar to our hypothesis can be discerned. This concordance between Burnet's treatment, primarily from the biological and immunological points of view, and the present independent elaboration from the direction of biochemistry, is reassuring, and full credit should go to Burnet for his prior publication. Differences do remain, however, particularly in the specification of the material elements involved and in the interpretation of the secondary response. Burnet, for example, suggests that Ab production occurs in the absence of Ag, whereas we hypothesize that Ag must be present as an inducer. This consideration of a dual action of Ag in two phases of the response parallels the earlier formulation by Dixon and associates ('52).

Jerne's ingenious treatment ('55) of this subject will not be elaborated here. His primary mechanism, the mobilization of Ag-Ab complexes at the site of prospective Ab synthesis, is on a different level from our consideration, and might even be incorporated at this level in our scheme. In the subsequent cellular processes postulated by Jerne, however, the absence of a known biochemical mechanism through which an Ab may direct the production of more protein like itself constitutes a serious deficiency. A most interesting treatment of the Ab response, related in part to the Jerne hypothesis, will be published by Talmage ('58).

The mechanism of Ab formation remains a challenging problem. Speculation has its main virtues in the suggestion of new and fruitful experimental approaches to the solution of this problem, and in bringing to bear upon it facts already available in various fields. The increasing lore of protein biosynthesis is among the most fertile sources of relevant facts.

SUMMARY

This paper was developed from a point of view provided by current information on the biochemistry of protein synthesis. Induced enzyme and Ab synthesis were treated as special aspects of the general mechanism, and it was sug-

gested that Ab formation involves a unique provision for modification by Ag of the heritable equipment of particular cells that can make globulins. Several possible bases for this modification and its cellular inheritance were mentioned, but an initial effect of Ag on nuclear DNA was selected as the simplest and most straightforward possibility, in terms of existing knowledge of the biochemical potentialities of cells in protein synthesis. Given a heritable change produced in DNA by Ag, and the consequent development of new RNP templates, a second action of Ag was suggested, very similar to the action of inducer in adaptive enzyme synthesis. At the immediate level of Ab production, therefore, Ag is postulated to act as a necessary inducer for Ab synthesis, in a system that has been conditioned for this response by an initial effect of Ag on DNA. Immunological observations appear to be consistent with this hypothesis.

OPEN DISCUSSION

PAPPENHEIMER[3]: If I understood correctly, Dr. Schweet suggested that Ag enters the nucleus, where it modifies DNA and thereby produces a specific genetic change in certain cells so that their progeny will continue to produce Ab even in the absence of Ag. This is a most interesting suggestion, but we must not forget that other mechanisms not involving changes in DNA or in genetic constitution can be postulated to explain Ab formation. Recent studies on induced or adaptive enzyme formation in bacteria have furnished us with a model of how the secondary response to an Ag can be explained without the necessity for assuming a hereditary modification of the cells concerned in Ab production.

Monod and his coworkers at the Pasteur Institute showed that two distinct specific systems are involved in enzyme formation by bacteria, either of which may be inducible. The first of these systems, which Monod calls "permease," is probably located at or near the cell surface and is concerned with the capture of specific inducer, its transport across the

[3] A. M. Pappenheimer, New York University College of Medicine.

cell membrane, and its concentration within the cell. The second is the enzyme-forming system itself.

β-Thiomethylgalactoside (TMG) is an excellent *inducer* of the enzyme β-galactosidase in *Escherichia coli,* although it is not a *substrate* for the enzyme. Rickenburg *et al.* ('56), using low concentrations of S^{35}-labeled TMG showed a prolonged lag before enzyme production reaches its maximum rate. When ''preinduced'' cells are washed to remove all traces of inducer and again placed in medium containing low concentrations of TMG, enzyme production starts immediately at the maximum rate. Preinduced cells containing the permease system can concentrate TMG about 100 times as effectively as uninduced cells.

The work from Monod's laboratory has further demonstrated that when inducer-free cells containing permease are allowed to grow in the absence of inducer, the permease system is divided up and diluted among the progeny; for several generations the progeny will then form enzyme at an accelerated rate when fresh TMG is added to them.

The response of preinduced bacteria to inducer is reminiscent of the secondary response of sensitized animals to a booster dose of Ag. I wished to remind you of the work with adaptive enzymes because it demonstrates how a specifically altered response can be transmitted through several generations without alteration of the genetic constitution of the cell. The work from Monod's laboratory showed that the permease-forming and enzyme-forming systems are under separate genetic control. Perhaps a similar situation exists in animals, and two gene-controlled inducible systems are involved—the first concerned with sensitization of the cell, capture of Ag, and its concentration at the site of Ab formation. The second genetically controlled system would be that involved in γ-globulin synthesis. According to this picture, the congenital agammaglobulinemic individual would be analogous to the bacterial mutant that lacks ability to form enzyme but is still capable of forming the corresponding permease. It has now been shown that delayed hypersensitivity can be readily induced in agammaglobulinemics to protein Ag that stimulate both hypersensitivity and Ab formation in normal individuals. Perhaps the delayed hypersensitive state represents something analogous to the permease system.

HAUROWITZ[4]: I always have to fight people who think that only RNA is necessary for coding protein formation. I am very glad that Dr. Novelli acknowledged that proteins are also necessary. I do not think we can explain protein synthesis without assuming that protein is involved in coding of new protein.

We have tried to investigate the RNA for amino acids as intermediates, since A. L. Dounce recently found amino acids in RNA. If these were intermediates, we should find the injected S^{35}-amino acid bound by RNA. We did not find any significant radioactivity bound to RNA.

Our autoradiographs show Ag localized in the cytoplasm, never in the nucleus. We find Ag in a zone around the nucleus, where it may influence the formation of proteins and Ab.

Dr. Owen mentioned the very interesting experiments of J. M. Dubert, in France, who used antigen A in the primary injection and antigen B in the second injection; the secondary response consisted in formation of anti-A. It is hardly possible to assume that this Ab is formed in response to the second injection. The first Ab continues to be formed slowly, and its formation is then accelerated by the second injection.

NOVELLI: A point that I did not bring out very clearly in my talk concerns the specificity of a given protein molecule. We can think of there being two orders of structural specificity in proteins, one determined by the sequence of the amino acids in the molecule and the other by the particular folding of the molecule. Theoretically, it would seem that a protein, consisting of a definite amino acid sequence, could be folded in a number of different ways to give molecules with different surface specificities but each with the same amino acid sequence. These two orders of specificity could be controlled by different materials in a given cell. In the theory proposed by Dr. Schweet, I think that all of the specificity in Ab synthesis resides in the DNA. Alternatively, we might consider that the DNA controls the amino acid sequence in a given Ab and that the Ag regulates the manner in which the Ab is folded to give a specific surface. Thus, in a cell, it may well be that there is a template-forming system that is in business to make γ-globulin, and that the specific folding of the molecule can be modified, depending on the nature of the Ag. A crucial point for this argument would be a complete analysis of the amino acid sequence of different Ab in a given individual. The amino acid sequence, according to this argument, should be the same in all Ab formed in a given individual; the specificity of the Ab would then be determined only by the particular folding. Up to now, analyses of the amino acid sequence in Ab indicate that at least the terminal pentapeptide is the same in a number of different Ab in the same individual. Although these analyses are in line with the present suggestion, they are still too meager to be definitive.

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[4] F. Haurowitz, Indiana University.

MAURER[5]: F. J. Dixon and I ('55) published information on the anamnestic type of reaction produced by injection of a cross-reacting Ag into rabbits previously immunized with bovine serum albumin (BSA). When the Ab level had decreased significantly, we reimmunized the rabbits with I^{131}-labeled HSA (human serum albumin), which we know can cross-react with anti-BSA. We observed an anamnestic type of elimination of I*HSA, an early appearance of anti-HSA, and simultaneously obtained an anamnestic type response against BSA.

The method of analysis was quite tricky and involved a number of cross-absorptions. Our data seem to fit in with Dr. Schweet's. In other words, by injecting a cross-reacting antigen, we could follow the mechanism that had been set up, so that upon injection of HSA, anti-BSA is produced that will not react with HSA. I do not know what system Dubert used, but if it involved a cross-reacting system the results obtained would not fit.

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[5] P. H. Maurer, University of Pittsburgh.

PAPPENHEIMER: I think it was dye-labeled serum albumin.

MAURER: These modified proteins were the subject of Dr. Kabat's thesis, in which it was shown that dye-labeled albumin can cross-react with antialbumin. One would therefore expect to obtain results like those of Dubert.

COONS[6]: I must say that on the basis of the histological evidence presented by other people, including my colleagues, I tend to associate myself with the idea of a modification of the heredity of this particular type of somatic cell. If we inject an Ag into the footpad of a rabbit and, within 24 hours, look for Ag, we can find thousands of cells that contain Ag scattered throughout the medullary areas of the lymph nodes around the follicles. Some of these cells, perhaps all of them, are cells that line the sinuses of the lymph nodes.

In Dr. Leon Weiss' studies of lymphoid tissue, under the electron microscope ('57), many of these reticular cells are present in lymphoid tissue in alternate rows between other cells, and between only a few of these opposing rows of reticular cells is there a channel. He concluded that these channels are open sometimes and closed at other times. One can imagine that, as Ag percolates through the lymph nodes, some cells become available to pick up Ag at one time, and others at other times.

We have begun to look at these cells more closely because we have started to test the hypothesis that an Ag is an Ag only if it can penetrate the nucleus of these cells and that materials unable, for chemical reasons, to get through the nuclear membrane cannot stimulate Ab formation. In our tests, we have found, in the lymph nodes of mice, reticular cells that contained five or six very small spots of BSA inside the nucleus with no other Ag in the neighborhood and apparently none in the cytoplasm.

[6] A. H. Coons, Harvard Medical School.

As I said, after 24 hours, many cells in the medullary area contain antigenic material. It is difficult to find Ag if we stain for it on the fourth day after injection. But if we now stain for Ab against this Ag, we can find in the whole section perhaps twenty-five cells that contain Ab. These cells are scattered. They are usually round. Once in a while they may be in pairs, but the contrast between the large number of cells that have taken up Ag by 24 hours and the small number that, during the primary response, make any Ab is very striking.

If time is allowed to pass after one injection of Ag—let's start with a 4-week period—and a second injection of Ag is given, the situation is very different. Here on the second day, traces of Ag can be found in very large cells with a large nucleus and a thin rim of cytoplasm, and sometimes there is a barely detectable amount of Ab in the cytoplasm. Sometimes we find sets of cells that have a few bright spots of Ab in localized areas in the cytoplasm, with no apparent Ab around them in the rest of the cytoplasm; and occasionally in such a large, primitive cell a structure that might be the nucleolus also contains some Ab.

If the secondary response is examined on the second day, no Ag is visible—we are just talking about Ab here. The contrast between the primary and the secondary responses is that now there are hundreds of cells, large, primitive ones, scattered throughout the medullary areas containing traces of Ab. These cells are undergoing rapid mitotic division, so that one of the principal reasons for the great burst of Ab synthesis during the secondary response is cell multiplication. We predict that the Ab response could be greatly modified by agents that alter the rate of cell division. These cells, during the next 2 days, multiply and differentiate. Their descendants, or several of them, form clumps of more-differentiated cells, which on the fourth day are mature plasma cells, and which multiply, differentiate, and synthesize a new protein that was not there before the second injection. Moreover, these large, primitive cells were not present in the lymph node before the second injection. They have arisen from some other cell as a result of the antigenic stimulus.

That is the picture at 4 weeks. If we shorten the interval between the two Ag injections, the number of these cells that respond to a large dose of Ag is smaller. In experiments with mice on production of diphtheria or tetanus antitoxin, Ipsen showed that the peak of the secondary response rises as the interval between the two injections increases. Therefore, the primitive cell that became sensitized or changed in some way by its first exposure to Ag but that did not make any Ab, apparently increases in number with time between the first and second injections.

This is consistent so far (although it does not establish it) with some genetic change in the cells that have become sensitized and do multiply, because the number of cells that can respond during the second injection increases as the time interval is prolonged, up to 3 or 4 weeks. Of course it has long been known that a good secondary response is not obtained in less than about 3 weeks. We do not know how far this curve goes up; and it is possible that particles are being distributed among daughter cells, particles containing the Ag. When the number of particles reaches 1% we would expect this curve to flatten out, but I do not think critical evidence differentiating between the required presence of Ag and the inheritance of some genetic change can be obtained by this kind of data.

PAPPENHEIMER: I think the problem here is whether they multiply after or before they become plasma cells.

COONS: They apparently do both. Two sets of mitoses are involved—one that we know about and one that is postulated.

OWEN: Our point of view on many of the matters raised in this discussion has been expressed in the paper, and need not be repeated. The suggestion that the continued presence of Ag is essential, to act as an inducer in the second phase of the Ab response, was derived from a consideration of adaptive enzyme formation, including the work by Monod discussed in more detail by Dr. Pappenheimer. With regard to the transmission of cytoplasmic changes, it is important to recognize that the nucleus places severe restrictions on what kind of change can occur and be perpetuated. For example, in serotype transformation in ciliates, it is true that different stable cytoplasmic states may be established without genetic change, as a function of different conditions of culture. But the kinds of changes that can occur are determined by the nucleus. A similar principle appears to hold in adaptive enzyme formation. In Ab elaboration, a very great diversity of potential for specific synthesis is evident. Analogy with adaptive enzyme formation led to the hypothesis that an initial effect of antigen on DNA, conferring a new nuclear potentiality, might underlie this great diversity. A distinction therefore may be drawn between the *secondary* response, in which Dr. Pappenheimer, like us, appears to implicate Ag as an inducer in a system that has been rendered inducible, and the *initial* effect of Ag that renders the system inducible and in which Dr. Pappenheimer also seems to recognize, in adaptive enzymes, a degree of genetic control. We limited our discussion to serum Ab, and did not venture into the field of delayed hypersensitivity to which he referred. In that field, it would seem important to distinguish between the analog of Ab and the machinery for producing it.

PAPPENHEIMER: There is one other mechanism of transfer, namely, that in which Lawrence used DNA-free extracts from peripheral white cells from sensitive donors. Transfer of delayed hypersensitivity to nonsensitive recipients by use of DNAse-treated cell extracts is of long duration. So that you have something here that can be passed on from one cell to another, which apparently does not multiply and which apparently is not DNA.

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DIVERSITY OF ANTIBODIES[1]

DAVID W. TALMAGE
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FOUR FIGURES

Typically, antibodies are recognized according to the qualities in which they are diverse. For convenience, these are classified according to primary properties and secondary reactivities. The primary properties are: (1) antigenic specificity, which in the most precise sense indicates the particular chemical or spatial configuration with which the antibody combines; (2) avidity for antigen, which is measured by the equilibrium constant of the reaction; and (3) physicochemical properties, such as size, shape, electrophoretic mobility, and amino acid sequence. Secondary reactivities include: (1) aggregating capacity; (2) complement-fixing capacity; and (3) biological activity. The last-named property refers to the cytotoxic or allergic responses resulting from the antigen-antibody union.

There is little reason to doubt that antibodies differ in all the respects listed. The question at issue is how these various differences relate to one another. Undoubtedly, one of the most important factors influencing all the other properties of an antibody is its antigenic specificity or the properties of the antigen to which it is bound. For example, the nonagglutinating or blocking property of certain Rh antibodies has been attributed to the location of the complementary antigen sufficiently deeply within the red cell surface structure to prevent the antibody molecule from linking two cells (Coombs *et al.*, '51). This possibility is borne out by the fact that treatment

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[1] This work was done with the support of Grant A-358 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health.

of the red cells with enzymes converts the blocking antibody into an agglutinating antibody (Pickles, '46). Similarly, it is possible to attribute the biological potency of certain antigen-antibody systems to the localization of the antigen in key shock organs or at selected vulnerable spots of certain cells. Since identity of specificity is difficult if not impossible to prove, almost any difference in antibody activity can be attributed to a possible difference in specificity (Bronfenbrenner, '48; Kabat, '51). For this reason, it has been difficult to challenge the position that the major factor determining antibody activity is its specificity and, for each antigenic configuration, only one antibody is formed that is responsible for all the observed manifestations of antibody activity. This hypothesis, sometimes called the "unitarian theory," has the merit of simplicity in design. There is an alternative concept of the nature of antibodies, however, that although more complex in design, explains more simply the known experimental observations. This hypothesis, which might be called the "multiple reaction theory," states that a given antigen and a given antibody may enter into a large number of different reactions, many of which may be competitive or even mutually exclusive. The manner in which a given antigen-antibody reaction manifests itself will depend on many factors including the avidity of the combination, the physicochemical properties of both the antibody and the antigen, the relative and absolute concentrations of the reactants, and the presence of auxiliary substances such as complement and mast cells. Selection of either antigen or antibody as all-important is unreasonable. If, for example, Rh antibodies fail to agglutinate the appropriate red cells, this failure cannot be attributed solely to localization of the reacting antigen, but must be the net effect of both antigen and antibody. The system could be made agglutinating by making the antigen more superficial or by selecting a larger antibody molecule. Whereas Ehrlich ('00) made the mistake of ascribing all the differences in the observed activities of antibodies to special attributes in the structure of the antibodies, the "unitarians" appear to have made the opposite mistake of attributing all the differences to the antigen. In any given system, the relative importance of the various factors can be determined only from a detailed knowledge of the mechanism of reaction.

In some instances one secondary manifestation of the antigen-antibody reaction will depend on another. For example, the ability of antigen-antibody systems to produce Arthus reactions appears to depend on their precipitating capacity (Kabat and Benacerraf, '49; Benacerraf and Kabat, '50); and their capacity to produce hemolysis may depend on complement-fixing capacity. In the hemolytic system, the physicochemical properties of the antibodies appear to play a prominent role. Figure 1 gives the results of an electrophoretic separation of a rabbit antiserum against the Forssman antigen of the sheep red cell (Stelos and Talmage, '57). This

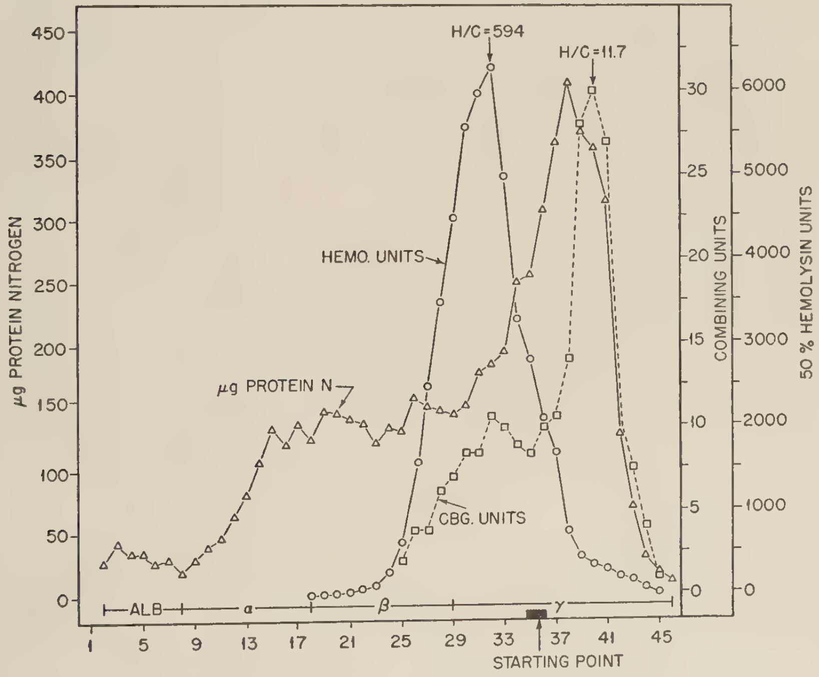

Fig. 1 Electrophoretic fractionation in starch of the globulin fraction of an antiserum to heated sheep red cell stromata.

fractionation reveals two distinct peaks of anti-Forssman activity, one on the fast- and the other on the slow-moving side of the γ-globulin fraction. The fast-moving globulins possess a ratio of hemolytic to combining units (H/C in figure 1) 50–100 times as great as the slow-moving globulins. This cannot be taken to indicate that electrophoretic mobility as such plays any role in the efficiency of the hemolytic process since these molecules also differ in size and perhaps in other respects. However, a number of reasons favor the idea that some physicochemical property is more important for the hemolytic function than antigenic specificity. (1) Adsorption tests in which varying mixtures of these two antibodies and guinea pig kidney as well as sheep cell Forssman antigen were used did not reveal any difference in specificity (Talmage *et al.*, '56). (2) Antiserums against an entirely different antigen of the sheep red cell, the isophile or heat-labile antigen, contained the same two peaks of antibody activity, and again the large and fast-moving molecule was more hemolytically efficient than the small and slow-moving molecule (Stelos and Talmage, '57). (3) Antiserums against adsorbed or chemically bound antigens have been shown to be capable of hemolyzing red cells (Fisher and Keogh, '50; Silverstein and Maltaner, '52). These three findings do not rule out entirely the role of the antigen as a factor in determining hemolytic efficiency. They do, however, make it difficult to explain the large observed differences solely on this basis. A fourth finding provides a possible mechanism by which the physical property of the antibody affects the hemolytic efficiency of the antigen-antibody reaction. A study by Weinrach and Talmage ('57) of the relation between antibody concentration and hemolytic rate indicates that some key reaction in the hemolytic process requires either two large or four small antibody molecules. More specifically, Weinrach's results showed that the hemolytic rate varied as the square of antibody concentration in the γ_1-globulin and with the fourth power of the antibody concentration in the γ_2-globulin. As a result of this finding, a model for the reaction between antibody and red cell in the hemolytic process would be as follows:

$$E + 2A \rightleftharpoons AEA$$
$$AEA \rightleftharpoons EA_2$$
$$EA_2 + C' \rightarrow \text{hemolysis}$$

In the first equation, the reaction between an erythrocyte (E) and two antibody molecules (A) results in the random distribution of the antibody molecules on the more than 1000-fold excess of antigenic sites available on the red cell surface. This reaction continues in equilibrium until by chance two antibody molecules combine with adjoining sites on the red cell to give the complex EA_2. The rate of formation of such complexes would be proportional to the square of the antibody concentration. The requirement of a larger complex such as EA_4 for the smaller molecule would explain both the observed fourth-power reaction and the decreased hemolytic efficiency of the smaller molecules. This hypothesis of the mechanism of immune hemolysis is strengthened by several considerations. First, the hemolysis of red cells, when complement was added before antibody, was a straight-line exponential decay, indicating a dynamic equilibrium in which the chance of hemolysis did not change with time. If, however, complement was added after antibody, hemolysis proceeded rapidly at first, indicating an accumulation of hemolyzable complexes. After part of the cells were hemolyzed, the rate of hemolysis then slowed until it reached a rate equal to that observed when complement was added first. From estimates of the number of antigenic sites per cell and the number of hemolysin molecules per cell, we could predict that antibody molecules would have to turn over or move from site to site at least once per minute to account for the observed hemolytic rate. When previously developed techniques of adsorption and elution were used (Talmage and Freter, '56), a labeled antibody was prepared that had adsorption characteristics nearly identical to hemolysin. After this labeled antibody was adsorbed on red cells, a large excess of unlabeled antibody was added and the rate of elution of the labeled antibody was measured. The results of this experiment are given in figure 2. Since mechanical trapping may slow down elution from the red cell, the observed half-elution time of three minutes agrees satisfactorily with the predicted turnover time of one minute.

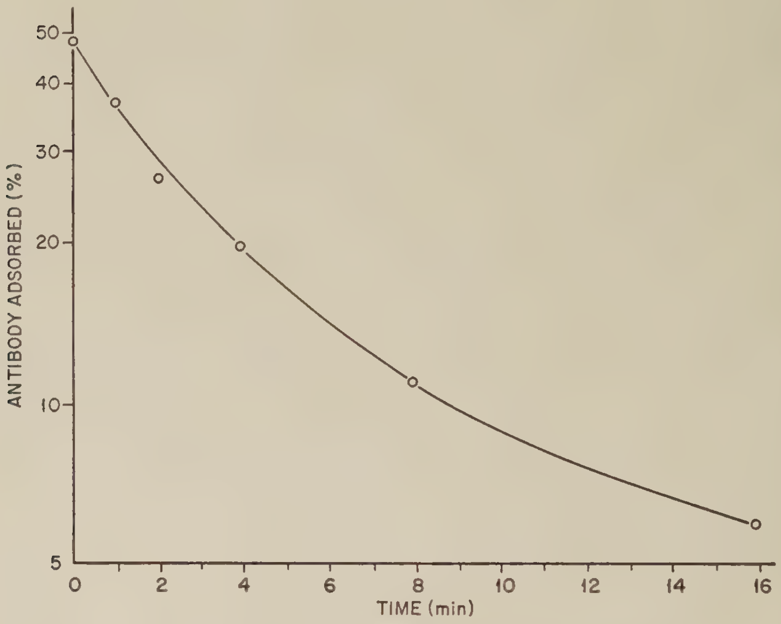

Fig. 2 The elution of labeled antibody from red cells in the presence of a large excess of unlabeled antibody. Half-elution time, 3 minutes.

The demonstration of the probable importance of avidity in the hemolytic process led to a search for better methods of measuring the equilibrium constants of antigen-antibody reactions. The existing methods of equilibrium dialysis (Eisen and Karush, '49) and light scattering (Epstein *et al.*, '56) required high concentrations of purified and thus-selected antibody from hyperimmune animals. At about this time, Farr ('56) developed his technique of using half-saturated ammonium sulfate to separate free labeled antigen from antigen bound to antibody. This is the only existing *in vitro* method of measuring equilibrium constants with primary response serums. Despite instinctive fears that the ammonium sulfate would alter the antigen-antibody equilibrium, no evidence of such an effect could be obtained. Addition of the labeled antigen and the ammonium sulfate to the antiserum simultaneously did not result in association of the antigen and antibody. Dissociation of complexes did not occur in the precipitating range, where complete or nearly complete binding of antigen takes place. Likewise, since Farr's method has given the lowest dissociation constants yet reported for antigen-antibody reactions, the ammonium sulfate probably does not produce significant dissociation.

By Farr's technique, a plot of antigen binding by primary and secondary response serums revealed striking differences in the two serums (fig. 3). With the primary or day-12 serum,

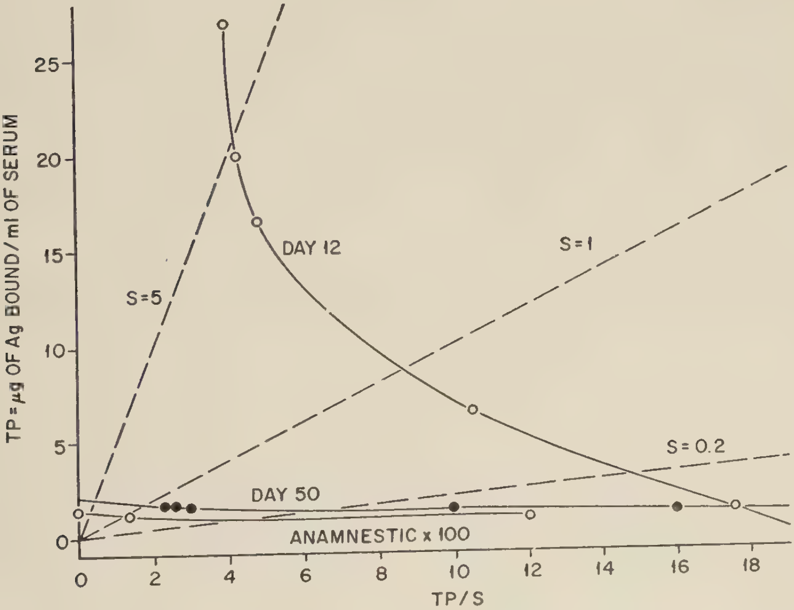

Fig. 3 A comparison of the antigen binding curves obtained with day-12 antiserum after the first injection of bovine serum albumin and with the anamnestic antiserum obtained after the second injection of antigen on day 50. S = free antigen concentration.

increasing concentrations of free antigen resulted in ever-increasing amounts of antigen bound to antibody. Since a homogeneous antibody population should give a straight line with this type of plot, the observed curvature indicates antibodies of varying affinity for antigen. The secondary response serum gave a straighter line, indicating greater homogeneity, and a flatter slope indicating a lower dissociation constant. One interesting result of the heterogeneity of combining constants is that the particular constant obtained depends to a considerable extent on the range of antigen concentrations used. Thus the higher the concentration of free antigen the steeper will be the slope of the plot and the higher the dissociation constant obtained in that range. In fact, the dissociation constant usually closely approximates the range of antigen concentrations used, although for secondary response serums it may be somewhat lower.

Table 1 lists the available data comparing observed equilibrium constants with the molar concentrations of the antigen used in the equilibrium experiments. For the purposes of this comparison the equilibrium constant has been expressed as the dissociation constant K_D, which in a homogeneous population is equal to the concentration of free antigen at which half of the antibody is bound. The table indicates a 10,000-fold difference in observed dissociation constants. The highest and lowest figures were obtained with rabbit antibodies to the

TABLE 1

Ration between antigen concentration and observed dissociation constant (K_D)

LITERATURE REFERENCE	ANTIGEN	MOLAR RANGE	OBSERVED K_D
Singer and Campbell ('55)	BSA[a]	$30–120 \times 10^{-5}$	4×10^{-5}
Lerman ('50)	H-Acid R	$10–100 \times 10^{-5}$	2×10^{-5}
Eisen and Karush ('49)	*p*-(*p*-HPA)-PAA	$5–80 \times 10^{-6}$	3×10^{-6}
Carsten and Eisen ('55)	DNP (precipitin)	$10–100 \times 10^{-6}$	5×10^{-6}
Carsten and Eisen ('55)	DNP (whole)	$10–100 \times 10^{-6}$	$3–30 \times 10^{-6}$
Farr ('56, '57)	BSA[a]	$6–60 \times 10^{-8}$	$0.60–60 \times 10^{-8}$

[a] Assumes valence of 6.

same antigen, bovine serum albumin. Since both the top and bottom figures make the same assumption that the valence of this antigen is 6, the magnitude of the difference cannot be ascribed to a valence artifact. The concept of a whole spectrum of antibody avidities first suggested by Jerne ('52) appears to be confirmed by these data. It is of equal interest that the table indicates an almost complete correlation between the concentration of antigen used and the equilibrium constant obtained. In each case, the dissociation constant is slightly lower than the molar concentration of antigen used. One result of this correlation is that the use of a restricted range of antigen concentration is likely to result in an unwarranted impression of antibody homogeneity.

The relation between antigen concentration and dissociation constant suggests a wide variety of antibodies for each antigen with an almost continuous spectrum of avidities and with a frequency distribution that increases progressively as the avidity decreases (dissociation increases, see figure 4).

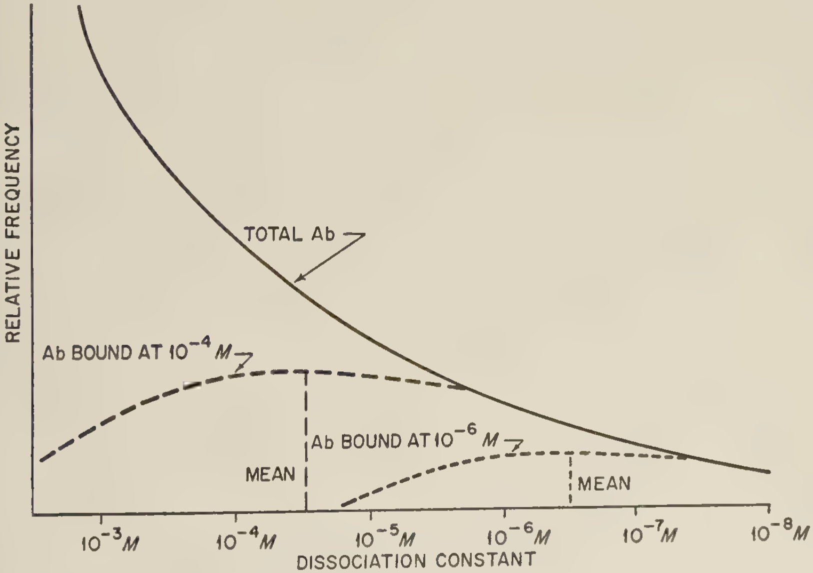

Fig. 4 Hypothetical plot of the distribution in serum of antibodies of varying avidity, and the amount of these antibodies bound at two antigen concentrations.

The solid line represents the hypothetical distribution of antibodies (Ab) in a serum. At any particular concentration of antigen, the following percentages of these antibodies are bound at equilibrium: 50% of antibody with a dissociation constant equal to the antigen concentration, 9% of antibody with a dissociation constant ten times the antigen concentration, and 91% of the antibody with a dissociation constant one-tenth the antigen concentration. The antibody bound at two different antigen concentrations is indicated in figure 4 by the two broken curves. As the antigen concentration is increased, antibody of lower avidity is progressively brought into the equilibrium and the average dissociation constant of the bound antibody progressively increases.

It is of interest that something like the frequency distribution illustrated in figure 4 was predicted by Pauling ('54) in a discussion of his theory of the formation of antibodies. According to this theory, antibody molecules that bind avidly to the antigen template probably are made more slowly than less avid molecules because of the time required to release them from the antigen template. There are, however, several difficulties with this theory. At the avid end of this antibody spectrum, the release time of the antigen-antibody complex is several days, at 37°C. and physiological *p*H and salt concentration. Yet more than 1000 and probably one million molecules of antibody can be produced per molecule of antigen injected (Haurowitz, '55). This approaches one molecule of antibody per second per template if all the antigen is assumed to be converted into templates. Although the first injection results in the production of relatively more nonavid antibody, a small second injection of antigen may be completely neutralized by the circulating antibody remaining from the first response and still give a greatly increased production of the most avid antibody.

At the other end of the antibody spectrum, the release time of the antigen-antibody complex is of the order of a second or less. No sharp line of demarcation exists between this antibody and the large mass of globulin that may be nonspecifically adsorbed to almost any antigen. This is a basic problem concerning antibodies — what they are and how they are produced. It is obviously impossible to draw an arbitrary line at any one point and to consider all dissociation constants below this point as antigen-antibody reactions and above this as nonspecific adsorption. If, however, the concept of absolute specificity is eliminated entirely, it is possible to conceive of antibodies as the tail in the normal frequency distribution of the diverse nonspecific bonds that form between antigen and naturally occurring globulins. This notion about the nature of antibodies is embodied in the natural selection theories of Ehrlich ('00) and Jerne ('55) and gives meaning to the diversity of antibody globulins, which was supposed to have been the subject of this paper. For it is only with a large and diverse population of globulin molecules that the tail in the normal distribution of nonspecific bonds could be as specific as antibodies generally are (Talmage, '57).

OPEN DISCUSSION

SINGER[2]: I would like to differentiate between "partition coefficients" as determined by the ammonium sulfate precipitation technique, and thermodynamic equilibrium constants for Ag-Ab reactions. A true equilibrium constant involves the concentrations, or activities, of single components; whereas in the partition technique, all the various Ag-Ab complexes that may form are lumped together as one component.

Concerning diversity of Ab, in our own studies the Ab is selected by its precipitability by Ag, and then by the solubility of the precipitate in excess antigen. This may have the effect of selecting a certain range of Ab "avidity," which may not represent the complete range that might be found in whole sera.

In connection with the kinetics of hemolysis being different with the large- and small-molecular-weight Ab, particularly with regard to the dependence of the rate of different powers of the Ab concentration, if the larger Ab had a larger valence

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[2] S. J. Singer, Yale University.

(4 or 6), it should attach more rapidly to the cell receptor sites simply on a statistical basis. This is a factor that would have to be considered in your model.

PAPPENHEIMER[3]: I think some of the old work on horse pneumococcus Ab suggested the existence of high-molecular-weight Ab with a valence of 12 or more. When S_{18} antibody is treated with pepsin, the large molecules are split into smaller Ab fragments, each of which has a valence of 2. The difference could therefore be much greater than between 2 and 4.

TALMAGE: The major factor determining the distribution of the various complexes to which Dr. Singer refers is the degree of Ag excess. This factor can be standardized in a labeled Ag system. For this purpose, we used an end point at which two-thirds of the antigen is free. Under these conditions, spontaneous aggregation does not occur and the fraction of complexes containing two Ab molecules is constant and negligible. There still remains the problem of a possible difference in the equilibrium constants of the first and second Ag molecules that combine with the two valences of a bivalent antibody. Although Singer and Campbell measured the equilibrium constant of the second Ag molecule, the results with the ammonium sulfate separation represent both equilibrium constants. If, for this reason, the term ''equilibrium constant'' is unacceptable thermodynamically, I am willing to accept Dr. Singer's suggestion that we call it a ''partition coefficient.'' I do not believe that this makes it any less valid a method of comparing the avidity of various Ag-Ab complexes. In the face of the tremendous heterogeneity of Ab combining sites, a single equilibrium constant obtained at a single Ag concentration has relatively little meaning.

Another point is that if one uses the dissociation constant that Dr. Singer obtained, something like 10^{-6} or 10^{-5} *M*, and the Ag concentrations that we have used, which are around 10^{-8} or 10^{-9} *M*, one can easily show that something like 0.001 or 0.0001 of this Ag would be bound at equilibrium. No matter

[3] A. M. Pappenheimer, New York University College of Medicine.

what name we give the constant obtained, the fact that we find one-third of the Ag bound at Ag concentrations of 10^{-8} *M* indicates that there is some Ab in the serum of much higher avidity than that studied by Dr Singer.

I would certainly agree with Drs. Singer and Pappenheimer that a difference in valence might explain some of the difference in hemolytic efficiency of the two antibodies. However, this would seem to be directly proportional to the differences in valence. Since we have something like a 100-fold difference in hemolytic efficiency and there are only 6-fold differences in molecular weight here, valence cannot account for the entire effect. In addition, a valence difference would have no direct effect on the relation between the concentration of Ab and the rate of hemolysis. I am referring to the finding that with one molecule the rate varies as the square of the concentration and in the other it varies as the fourth power of the concentration.

PAPPENHEIMER: Perhaps Dr. Singer might be interested in antisera containing Ab of known avidity for study of equilibria in the ultracentrifuge. The system most studied has been the diphtheria toxin-antitoxin reaction in the horse. According to the British, their antitoxins are very much more avid than those produced in this country and a comparison might be interesting.

SUSKIND[4]: If, as Dr. Schweet and Dr. Owen propose, the role of the Ag is to modify the DNA in certain Ab-forming cells, and if we assume that a one-gene-to-one-protein relation obtains in these cells, then it might be expected that the probability of two such alterations occurring in a single cell would be extremely small.

Would it be possible to immunize with two unrelated Ag, and using Dr. Coons' fluorescent antibody technique, determine whether antibodies against both antigens are formed in a single cell?

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[4] S. R. Suskind, McCollum-Pratt Institute, The Johns Hopkins University.

Coons[5]: We have done one experiment on four rabbits on this point. We injected into the same footpad a mixture of ovalbumin and diphtheria toxoid, waited 4 weeks so we could study the secondary response, and reinjected the same mixture of antigens. Then we cut serial sections of this lymph node. Unfortunately, at that time we had only one fluorescent label so we could not study the presence of these two antibodies in the same section. But we could stain one section for diphtheria antitoxin, another for ovalbumin, and a third for both. We then found clusters of cells in the lymph node containing diphtheria antitoxin. There were about the same number of clusters containing ovalbumin and about twice as many clusters when we stained for both antibodies at the same time.

This means that in general these cells were making either one kind of Ab or the other. It does not exclude the possibility that there were some cells engaged in synthesizing both antibodies.

Pressman[6]: The term "avidity" is being used very loosely to include equilibrium constant and rate effects. These are two different properties. The equilibrium constant k may be taken as the ratio of the forward and reverse rates of reaction, but it is a definite value quite separate from the rate of reaction in one particular direction. I have been confused by the use of avidity to mean rate of dissociation in one case and equilibrium in another.

Then I should like to take up the point of second- and fourth-order reactions for the action of the hemolytic antibodies. A high order of reaction will explain a very steep change with concentration; yet in a system that has heterogeneous combining constants, such as you have, a fourth-power term may be effective in smoothing out a curve for a short range of concentration changes.

I think that we have to look at such values with great caution. The values should be determined over large changes, in concentration, and I think there is a limitation in the system itself in whether these large changes can be handled before the reaction gets out of hand.

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[5] A. H. Coons, Harvard Medical School.

[6] David Pressman, Roswell Park Memorial Institute.

Anderson[7]: It bothers me somewhat that the assumption has been made all morning that we know something about DNA or RNA as templates, whereas experimentally no one has ever demonstrated that any particular DNA or RNA preparation is a template for any specific thing. This is yet to be done.

Another matter is in connection with the S_{17} or S_{20} proteins. It is very interesting that in the rat, where, unfortunately, we have not done any immunological work with this heavy fraction, this heavy component looks very much like the γ-globulin if we observe it by paper electrophoresis. It does not move. But if we study it in free electrophoresis, it stays right out with the alphas, and I suspect that perhaps if this material were prepared in starch it might not move either. I think this material should be studied in free electrophoresis to find out whether it actually is a gamma or an alpha. Certainly we ought to see it as a sharp peak whereever it is.

Farr[8]: I should like to clarify just one point about this k with ammonium sulfate. We don't believe this is a true k in the sense of salt solution or in a true physical-chemical sense, and we don't want to propose that we think it is. Two things are wrong with it. In the first place, we don't know the effective valence of Ag in solution. Although with extreme Ab excess, we can get an estimate of the minimum number of Ag sites on the BSA molecule, we don't know how many antigenic sites there are on a free-floating BSA molecule. The other difficulty is the heterogeneity of Ab in the antisera and the inability to get a uniform population of Ab to measure. I think that these two considerations preclude use of whole serum as a source of Ab in an attempt to give a real k value with the ammonium sulfate or any other method. On the other hand, the value obtained is related to the average strength of the Ag-Ab bond and is useful as a measure of avidity.

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[7] N. G. Anderson, Biology Division, Oak Ridge National Laboratory.

[8] Richard Farr, University of Pittsburgh.

TALMAGE: I don't want to add anything to what Dr. Farr just said, but I should like to comment on Dr. Pressman's criticism of the relation between rate and concentration.

If we vary the concentration in the small molecule as much as twofold, we obtain a 16-fold change in rate and this approximately equals the ratio of the upper and lower limits at which hemolytic rates can be studied. The most rapid hemolytic rate that can be measured accurately is obtained with a concentration of hemolysin that gives 50% hemolysis in 10 minutes. With one-half this concentration of Ab, 50% hemolysis is obtained in 160 minutes. With slower hemolysis there are problems of complement decay. The limiting factors are there but I would still feel that a 16-fold change of rate in a twofold change of concentration provides a fairly accurate estimate of the way that rate varies with concentration.

In answer to Dr. Pressman's comment about the looseness of the meaning of the term ''avidity,'' there is a need for terms of varying precision of meaning. I do not believe that at the present time we should equate avidity with any exact thermodynamic constant.

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ADOPTIVE TRANSFER OF IMMUNE REACTIONS BY CELLS

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TWO FIGURES

The immune state may be acquired actively, by the introduction of antigen into a suitable animal. It may also be acquired passively, by the introduction of antibody prepared in another animal. To these two classical categories, a third has now been added — an immune state transferred from one animal to another by immunologically activated cells. Billingham, Brent, and Medawar ('54) have named the state of immunity acquired in this way "adoptive" immunity.

Transfer of immune reactions by cells is in no sense new. Deutsch (1899) injected spleen tissue from guinea pigs actively immunized against typhoid bacilli into other guinea pigs and found a transient appearance of antityphoid agglutinins to a low titer. Luckhardt and Becht ('11) carried out similar spleen cell transfers in the dog. These were followed by Topley's work ('30) with the rabbit, in which careful allowance was made for the concomitant active and passive immunization. Later studies have extended the range of experimental animals and antigenic stimuli, and varied the experimental design (references are given by Billingham, Brent, and Medawar, '54; Mitchison, '55; Dixon and Weigle, '57).

REACTIONS MEDIATED BY CELLS

A new type of immune reaction was identified when Landsteiner and Chase ('42) showed that sensitivity of the delayed type to simple organic compounds could be transferred by cells, but not by serum. Other types of immunity that have been transferred by cells alone are sensitivity to tuberculin, various forms of "allergic eczema," tissue transplantation immunity, and perhaps allergic autoimmunity (Lipton and Freund, '53; for other references see Billingham, Brent, and Medawar, '54). The conclusion that these reactions are mediated by cells is supported by other evidence, such as their failure to act across membranes impermeable to cells (Algire *et al.*, '55).

This type of immunity is particularly valuable in demonstrating the role of the cells in conferring immunity on the host, since formed antibody transferred with the cells is without effect.

Metaxas and Metaxas-Bühler ('55) have injected cells from guinea pigs sensitized to tuberculin intravenously into other guinea pigs, which then will react to tuberculin. The cells appear to participate directly in the reaction of the host, without first becoming established in the host tissues, synthesizing or liberating antibody, or giving rise to progeny. Evidence for this is the absence of any latent period between transfer and the host's becoming sensitive; and also that in animals given minimal amounts of cells, the reactions to multiple intradermal test doses are markedly weaker than the reaction to a single dose. In this case it would seem that the cells are playing a passive role, similar to that of serum antibody in passive immunity. This type of transfer differs therefore in principle from the transfer of antibody-producing cells, wherein the synthesis of new material in the host is an essential feature.

THE SITE OF ANTIBODY PRODUCTION

Different routes of injection stimulate different sites of antibody production. For example, intradermal or subcutaneous injection of antigen stimulates the regional lymph nodes, as has been shown by the concentration of antibody in the tissue (McMaster and Hudack, '35) and in the draining lymph ducts (Ehrich and Harris, '42). The activity of such tissue when transferred is in good agreement with this evidence. The lymph nodes draining the site of tissue homografts are the most active in transferring transplantation immunity (Mitchison, '53, '54, '55; Billingham, Brent, and Medawar, '54), and the popliteal node is most active in transferring antibody production after injection of bacterial antigen into the foot pad (T. N. Harris *et al.*, '54). Some activity of the spleen is also detectable by transfer in these cases, but this organ is best stimulated by intravenous injection (Stavitsky, '54).

After antigen is injected into a tissue, time must be allowed for the antigenic stimulus to reach the regional lymph nodes. With a transplanted tumor, this time is 3–5 days (Mitchison, '55). During the secondary response to transplants of the tumor, the lymph nodes are activated more rapidly, as might be expected from the earlier breakdown of the transplants. With dysentery organisms the time from the foot pad to the popliteal node can be as short as 10 minutes (S. Harris *et al.*, '54).

Transfer experiments have been more valuable in locating the organ than the cell type responsible for antibody production. This is partly because the cell populations of the lymph nodes, spleen and peritoneal exudates are heterogeneous. More homogeneous populations of plasma cells have been obtained from the omentum (Roberts, '55). A further difficulty is that the proportion of active cells among those transferred is unknown.

TRANSFER OF CELLS INTO NONREACTIVE HOSTS

Billingham, Brent, and Medawar ('54) made use of skin homografts on tolerant hosts to show that lymph node cells can be activated immunologically after transfer. These skin homografts are accepted and incorporated into the hosts, but can be destroyed by intraperitoneal inoculation with lymph node fragments from mice of the host strain. They can also be destroyed by inoculation of normal lymph node tissue derived from mice of the same strain and that had not been actively immunized. The tempo of the reaction brought about by normal lymph nodes is much slower. Presumably, normal lymph node cells become incorporated into the host, and respond to a skin graft by a process of active immunization.

The agammaglobulinemic human patient, as well as the tolerant mouse, can be equipped with antibody-producing lymphoid cells. In agammaglobulinemics, the transfer of lymphoid cells from normal human beings who have been hyperimmunized with typhoid vaccine and diphtheria toxin results in significant production of specific antibody for two to three months (R. A. Good, cited by Dixon and Weigle,' 57).

THE LOCATION OF CELLS AFTER TRANSFER

Immunologically activated cells may be injected into the host by a route designed to produce a local state of immunity. Thus Metaxas and Metaxas-Bühler ('48) injected peritoneal exudate cells from guinea pigs sensitized to tuberculin into the skin of other guinea pigs. The hosts exhibited sensitivity to tuberculin, but only at the prepared site. Sensitization of the skin of the guinea pig to rabbit spinal cord has been accomplished by cellular transfer in a similar way (M. W. Chase, J. Freund, and M. M. Lipton, cited by Lipton and Freund, '53). Localization at the site of injection no doubt also accounts for the failure of Billingham, Brent, and Medawar ('54) to transfer transplantation immunity by subcutaneous injection of lymph node cells.

When lymphoid cells are injected intravenously or intraperitoneally, the question arises whether they become incorporated in the host tissues at random or localize in homologous organs. A homing instinct may be expected for general reasons. One reason is the remarkable ability of bone marrow and immature spleen cells to colonize radiation-damaged homologous tissue (Lindsley *et al.*, '55; Mitchison, '56; Ford *et al.*, '56). Another reason is the probability of a recirculation of lymphocytes from the lymph nodes to the blood stream and back, suggested by the important new evidence of Mann and Higgins ('50) and Gowans ('57).

Localization of immature spleen cells in the spleen after intravenous injection in normal adult mice has been demonstrated by the use of isoantigenic markers (Mitchison, '56); similar localization follows intravenous injection of adult spleen cells into newborn mice (R. E. Billingham, unpub.). Some cells, at least, probably enter the circulation after injection into the abdominal cavity, presumably through the lymphatic vessels of the diaphragm (Courtice *et al.*, '53). The passage of lymph node and spleen cells out of the abdominal cavity into the host organs has been followed by vitally staining the cells with the fluorescent dye, acriflavine (Mitchison and Dube, '55; M. Holub, unpublished). About 20 hours is needed for this in the guinea pig, as may be inferred from the time taken to establish cutaneous sensitivity, after the intraperitoneal inoculation of sensitized cells (Metaxas and Metaxas-Bühler, '55).

A comparison of the effectiveness of the intravenous and intraperitoneal routes supports the hypothesis that cells function best after transfer if they become established in homologous host tissue. The intravenous route is the more effective for antibody-producing cells (S. Harris *et al.*, '54), as well as for bone marrow cells after irradiation (Kaplan *et al.*, '53).

ACTIVATION OF HOST CELLS

According to the interpretation so far considered, the transferred cells are solely responsible for the immune response of the host. Another possibility is that the cells of the host also participate in the response, presumably by taking in some subcellular fraction from the transferred cells. This possibility would need strong experimental support for its acceptance, because of the general evidence that large, specific molecules cannot enter tissue cells. An analogy to subcellular transfer may be found in the transfer of genetic material between microorganisms; and the studies of Hamilton ('56) and Billingham *et al.*, ('56) suggest that nucleoproteins can enter lymph node cells. Hamilton injected adenine 8-C^{14} into leukemic human patients and found out that the isotope was retained in the nucleic acids of lymphocytes for very long periods. This may mean that dying lymphocytes pass large fragments of nucleic acid or nucleoprotein on to their successors. Billingham, Brent, and Medawar showed that nucleoprotein extracts of tissue cells can provoke transplantation immunity, presumably by entering the cells of the regional lymph nodes.

Most of the direct evidence indicates that the cells of the host are not activated (reviewed by Billingham, Brent, and Medawar, '54; Mitchison, '55; Dixon and Weigle, '57):

Viability of transferred cells. Killed cells do not transfer immunity. This is so even with such mild agents as freezing, mild heat, and X radiation. An exception is the ability of disintegrated cells to transfer delayed sensitivity (Jeter *et al.*, '54; Lawrence, '55); but in these cases, there is no evidence that the transferred material initiates antibody production by the host cells.

Time of onset of antibody production in the host. Cells may be transferred from an immunized donor during the latent period between injection of antigen and the appearance of antibody. Antibody then appears in the host at a time related to the interval after active immunization of the donor, not the interval after transfer.

Suppression of antibody production by the homograft reaction against the transferred cells. The evidence for this is discussed in the next section.

Transfer of immunity into nonreactive hosts. Immunity has been transferred by cells into hosts that are themselves nonreactive for any one of the following reasons: X irradiation, actively acquired tolerance, pretreatment with lyophilized tissue, or immaturity (Šterzl, '55).

Quality of antibody. When cells have been transferred from donors secondarily stimulated with protein antigen, the antibody produced in the host has the combining power characteristic of the secondary, not the primary, response.

A claim to have transferred either an antibody precursor, or a cell-free system capable of producing antibody, has been made by Šterzl and Hrubešová ('56). Nucleoprotein was extracted from the spleen of immunized adults and inoculated into young rabbits, where transient agglutinin was detectable in the serum. The crucial point is whether the nucleoprotein preparation was free of preformed antibody: this has not been established beyond dispute.

THE HOMOGRAFT REACTION AGAINST TRANSFERRED CELLS

Adoptively acquired immunity generally declines after a period of days or weeks, and eventually disappears. It is worth while examining how far this decline can be accounted for by the homograft reaction; that is, the immune reaction of the host against the foreign antigens of the transplanted cells. Direct demonstration of a reaction against transplants of spleen or lymph node is difficult because of the complex histological structure of these tissues. But these tissues can elicit a strong immunity, detectable by heightened resistance against subsequent grafts of skin or tumor (Mitchison, '56; R. E. Billingham, L. Brent, and N. A. Mitchison, unpublished).

Tissue can be freely transplanted between individuals that are sufficiently closely related. On this principle, transplants of immunologically activated tissue have been made between mice of an inbred strain. In certain cases, the immunity adoptively acquired in this way declines no more rapidly than active immunity (Billingham, Brent, and Medawar, '54; Mitchison and Dube, '55); and the exceptions are probably caused by insufficient inbreeding.

An animal that has rejected a tissue transplant is more resistant to a second transplant. Thus first grafts of skin and certain tumors survive about 10 days on transplantation between mouse strains, whereas second grafts survive less than 6 days (Mitchison, '54, '55; Billingham, . . . , and Sparrow, '54). Consequently, when immunologically activated cells constitute a second transplant, the adoptive immunity that they produce may be expected to show a relatively rapid decline.

This expectation has been tested with a *Salmonella typhi* (H)-mouse spleen transfer system, by the methods and antigen dosage of Mitchison ('56). The following experiment demonstrates the general characteristics of this system. Antigen was given to CBA mice in two intravenous injections 20 days apart, and the spleens were taken 6 days later. Each spleen was minced and inoculated intraperitoneally into an A strain host; three hosts received living spleen tissue, three received spleen cells killed by freezing and thawing, and three others received an intraperitoneal injection of 2 ml of homologous antiserum. Subsequent antibody titers in the host serum are shown in figure 1. Except on the first day after transfer, the mice that received living cells had a higher titer of antibody than those receiving dead cells and, the titer continued to rise until the fourth day. The difference can be attributed to the production of fresh antibody by the living cells after transfer. After 4 days, the decline in the living cell host was not significantly different from the decline of passive immunity, indicating that cells stopped producing antibody at this time.

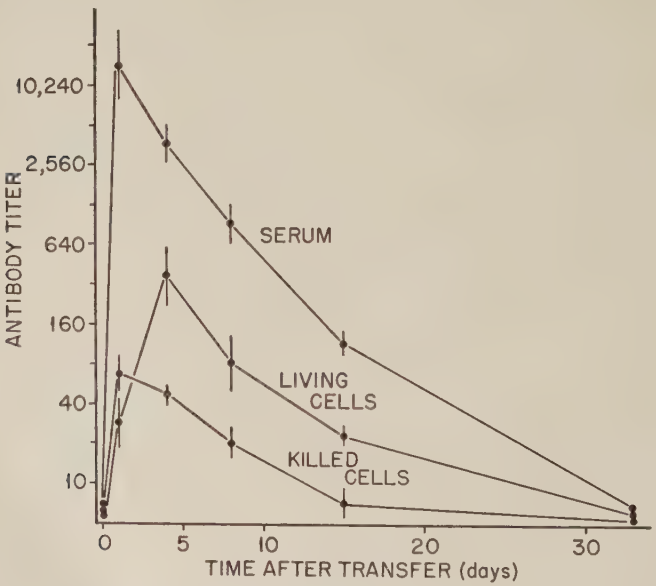

Fig. 1 Means and standard deviations of antibody titers after spleen cell transfer from CBA into A strain mice.

The same system was used to show the outcome of transfer within an inbred strain, between strains, and between strains after prior isoimmunization. CBA mice received the antigen at intervals of 7, 5, and 4 days. Their spleens were transferred 6 days later into 4 groups of hosts, each of 5 mice.

CBA hosts (*intrastrain*). To check the toleration of the transferred spleen cells, we transplanted skin grafts from the donors to the hosts 27 days before the transfer. Transplantation was made according to the fitted Thiersch graft technique of Billingham and Medawar ('51), and at no time throughout the experiment was any reaction against the grafts detectable.

Irradiated CBA hosts (*intrastrain*). A dose of 500 r of X radiation was given immediately before spleen cell transfer, at 240 kv, 15 ma, and half-value layer of 1.2 mm of Cu. Discussion of the outcome in this group is postponed until the next section.

A hosts (*interstrain*). Untreated.

Isoimmunized A hosts (*interstrain*). Six days before transfer, these hosts were inoculated intraperitoneally with about 40×10^6 CBA spleen cells. This dose is sufficient to produce a high degree of transplantation immunity (Mitchison, '56).

Antibody production hardly declined in the intrastrain hosts over the period of observation (fig. 2). Indeed, 4 months after transfer, the titers fell, by about two serial dilutions only. Production appears to have stopped in the nonpretreated interstrain hosts after about 5 days, as in the previous experiment. The decline started after one day in the interstrain hosts previously isoimmunized; in these hosts the transferred cells produced no more effect than the killed cells in the previous experiment.

These results fully support the hypothesis that spleen cells transferred between strains succumb to the homograft reaction. The homograft reaction can reasonably account for the eventual termination of adoptive immunity that is encountered in most laboratory animals, and in man.

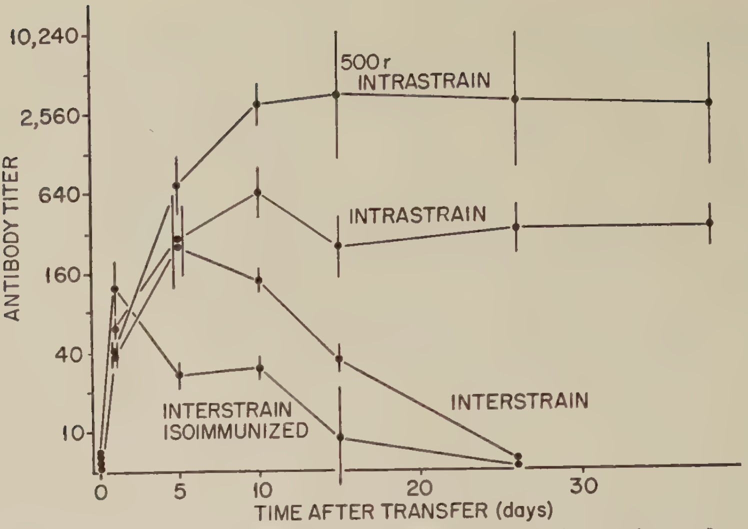

Fig. 2 Means and standard deviations of antibody titers after spleen cell transfer from CBA donor mice.

If this is the case, immunity to disease may be transferable between inbred mice by cells. Immunity to tuberculosis has not been transferred by serum, even in the careful experiments of Raffel ('55). The difficulty in designing an experiment to test for transfer of immunity by cells is that the survival of the cells is generally shorter than the incubation period of the disease. Experiments have indicated that immunity to tuberculosis can be transferred by cells within the CBA strain (D. A. Mitchison and N. A. Mitchison, unpublished).

CELL MULTIPLICATION AFTER TRANSFER

It is not known whether the antibody-producing mechanism in a cell duplicates itself as the cell divides. The more orthodox view is that determinant groups of the antigen must be retained in the antibody-producing cell (Pauling, '40; Haurowitz, '52; but see also Burnet and Fenner, '49). In this orthodox view, the capacity to manufacture antibody would be diluted out as the cells multiplied.

Compensatory growth suggests itself as a means of multiplying lymphoid cells. This phenomenon is exhibited after total surgical lymphadenectomy (Sanders and Florey, '40), when lymphoid tissue regenerates from minute tracts in the liver. Lymphoid tissue may more conveniently be destroyed by radiation. After potentially lethal irradiation, made sublethal either by shielding of the spleen or by subsequent cell transfer, lymphoid tissue and bone marrow regenerate from "spotty foci" (Jacobson, '52). The minimum dose of transferred cells reported to save a lethally irradiated mouse is one-fifth of a baby mouse spleen (Barnes and Loutit, '54), but smaller doses can apparently be effective (D. W. H. Barnes, unpublished). If these cells are to recolonize their host, they must multiply about one thousandfold.

In experiments with rabbits, Harris, Harris, *et al.*, ('54) showed that prior irradiation of the host enhances the production of antibodies by transferred lymph node cells. Unfortunately, this result is ambiguous, since the enhanced production may be attributed to suppression of the homograft reaction against the transferred cells. This ambiguity was avoided in the experiment reported in the previous section. The controls for the irradiated CBA hosts were the nonirradiated CBA hosts, in which the tolerated skin grafts show that no reaction has taken place against the transferred spleen cells. Antibody levels were significantly higher after irradiation, by a factor of about 8. The difference may be attributed to the action of the radiation in destroying the lymphoid tissue of the host, so leaving space for the transferred cells.

At the dose of radiation used, some host lymphoid cells survive and are capable of regenerating new tissue. Since the transferred cells carry no marker except their capacity to produce antibody, the extent of their multiplication cannot be estimated or correlated with antibody production.

There are indications that the chicken embryo may also be valuable as a site for the multiplication of antibody-producing cells. The work of Murphy ('16) suggests that spleen tissue can function immunologically after transplantation onto the chorioallantoic membrane. It has been shown repeatedly that material passes from grafts of adult chicken spleen on the membrane into the spleen of the embryo, which consequently enlarges (for references, see Ebert, '51). A new approach to this problem was suggested by the work of Hraba ('56). Chicken eggs were placed in parabiosis with turkey eggs and a chimera was obtained having both turkey and chicken erythrocyte antigens.

In the present preliminary experiments, grafts of adult turkey spleen have been transplanted onto the chorioallantois, or into the coelom, of chicken embryos. This results in enlargement of the embryo spleen, accompanied by the presence of turkey erythrocytes in the circulation, detectable by agglutination. At least some of these erythrocytes are manufactured in the embryo spleen, for retransplantation of the embryo spleen into young eggs again produces chimerism. At least $\times 10^4$ multiplication of the turkey erythropoietic tissue was obtained in 26 days. Using the same system, we detected agglutinins in the embryonic circulation after transplantation of spleen from adult chickens immunized against *S. typhi* (H).

OPEN DISCUSSION

Makinodan[1]: An interesting observation about circulating and noncirculating antibodies was made recently by Egashira of the Japan National Institutes of Health. He reported that induction of the delayed type of hypersensitivity in normal guinea pig was accomplished by injecting lyophilized and concentrated sera of guinea pigs with the delayed type of hypersensitivity.

Mitchison: Although transfer can be brought about by transferring a great deal of serum, this is not proof that serum is, in fact, the way the reaction is mediated in the normal animal. If you raise the titer of the serum antibody to the level that it is in the normal immunized animal without producing sensitivity or transplantation immunity, this is evidence that even though serum antibody can be shown to be effective, it is not normally so.

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[1] T. Makinodan, Oak Ridge National Laboratory.

RAPPORT[2]: Dr. Mitchison, is the duration of the adoptively acquired immunity in any way related to the quantity of lymph nodes that you administer, and can you continue it by continuing to administer lymph nodes?

MITCHISON: I don't think we have any evidence on that point.

SALK[3]: I don't know whether this is pertinent to this discussion, but this is reminiscent of the question that comes up repeatedly of the relation between neutralizing antibody and complement-fixing antibody, and of the relation of tissue or cellular immunity to humoral immunity.

We have just made some observations with a cell derived from a monkey heart tissue that now grows in continuous culture. When this cell is returned to the monkey subcutaneously, tumors develop in about one of twenty animals that look very much like the ones that were shown in the photographs here. In such animals, the one out of 20 variety, the tumor will develop to quite a large size, but at the height of the tumor development, which occurs in about 6 weeks (after about 90 days the tumor disappears), one can demonstrate the presence of an anti substance in the serum that can be detected in tissue culture.

If you mix the serum and these cells in culture, you can demonstrate a toxic effect on the cells. The cells will fail to grow. The interference with cell growth manifests itself in two ways, one that can be seen cytologically and the other that can be distinguished by a suppression of metabolic activity manifested by a reduction in the capacity of the cells to convert phenol red to the yellow form.

We observed that when these animals are reinoculated they fail to develop the tumor the second time, just as in this case, but if you were to try to passively transfer this immunity by means of serum you would fail to do so. You can, however, demonstrate the presence of humoral substance in the tissue culture tube, where the concentration of the reagents that are used can be controlled.

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[2] M. M. Rapport, Sloan-Kettering Institute.

[3] J. E. Salk, University of Pittsburgh School of Medicine.

That this is pertinent to the question of whether one is dealing purely with a cellular reaction or with activity of humoral substance to such cells is the reason I was bold enough to bring this up.

KOLLER [4]: I would like to add confirmatory evidence to the findings of Dr. Mitchison. Dr. Simonsen from the Fibiger Laboratories, Copenhagen, recently visited us and gave us a very interesting talk on the ''spleen-enlarging principle'' in homologous chimeras of chicken. Simonsen transferred cells from the spleen of 25-day-old chicken into 18-day-old chick embryo by intravenous injection. Three days after the chicks hatch, Simonsen found enlarged spleen in these young birds. The blood from chicks showing spleen enlargement was injected into 18-day-old chick embryos. Three days after hatching, the chicks that received only blood also had enlarged spleens. The process was repeated 9 times, and there was no dilution of the ''spleen-enlarging principle.'' Simonsen concluded that the donor cells from the spleen of 25-day-old chicks invade the spleen of the embryo and undergo multiplication, and this results in an increase of spleen size. Many of the donor cells enter into circulation, and can be transferred with blood into another embryo. Simonsen's observation ('57) confirms Dr. Mitchison's findings.

SCHREK [5]: I agree with the speaker that the cellular immunity is very important, but we ought not to overlook that in some cases humoral immunity plays a role. In some experiments, I used the transplantable Bagg rat tumor, which regressed in 25% of the rats. The homologous sera from these rats were able to kill the Bagg tumor cells. The death of the tumor cells could be demonstrated with time-lapse movies (Schrek and Preston, '56) and with unstained cell counts (Schrek and Preston, '57). It is evident that this tumor, at

[4] P. C. Koller, Chester Beatty Research Institute, London.

[5] R. Schrek, Northwestern University.

least, induced in homologous animals an antibody that, with complement, was cytotoxic.

ANDERSON[6]: I asked Dr. Medawar about contamination with granules. He does not get any effect if he uses concentrates of purified mitochondria. These may have been contaminated with a few nucleoprotein particles, but he got no positive effects when he used them.

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[6] N. G. Anderson, Oak Ridge National Laboratory.

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THE CELLULAR BASIS FOR ANTIBODY FORMATION [1]

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SIX FIGURES

The immunochemical aspects of antibody (Ab) formation have received considerable attention recently. Theories have been proposed explaining, primarily in biochemical terms, how an antigen (Ag) stimulates protein synthesis, how the remarkable specificity of the Ab is developed, how Ab formation is continued long after Ag injection, how Ab formation is depressed by X radiation and other agents, and how the Ab is released into the blood. Frequently these theories and their supporting evidence have been considered as involving one static cell type that presumably ingests or absorbs the Ag, synthesizes the Ab, actively releases it, and then returns to a resting state (Breinl and Haurowitz, '30; Alexander, '32; Mudd, '32; Pauling, '40; Dixon *et al.*, '52; Haurowitz, '53; Burnet and Fenner, '49; Jerne, '55; Boyd, '56; Talmage, '57).

The evidence presented in this paper suggests that at least four cell types are active in various stages of Ab formation. One type, the macrophage, ingests and digests a particulate Ag whereas another cell type, apparently not derived from these phagocytes, proliferates, gradually differentiates, and presumably initiates Ab synthesis. Near the end of the period in which Ab is formed, most of the cells of the latter type appear to be transformed into a small cell that superficially,

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at least, resembles a small lymphocyte and a few appear to develop into typical mature plasma cells. Under the conditions of these studies, then, both cell proliferation and differentiation seem to characterize the Ab-forming process. Further cell transformation and mobilization have been observed in the period that follows rapid Ab synthesis.

It seems likely that proper chemical interpretation of the process of Ab formation may be facilitated by an understanding of the cellular changes involved.

THE ROLE OF CELLS

The macrophage, lymphocyte, and plasma cell each have been thought to take part in Ab formation. The importance of each, frequently to the exclusion of the others, has been supported by numerous investigations (Wilson and Miles, '46; Fagraeus, '48; Taliaferro, '49; McMaster, '53; Boyd, '56).

The primitive reticular cell,[3] which under most conditions does not appear to be phagocytic (Maximow and Bloom, '52), is probably a precursor of all the cells implicated in Ab formation.

The experimental observations that form the basis for this paper demonstrate how the Ab-forming cell is involved in a dynamic series of cell transformations. With this concept, many cell types become important and interrelated in the Ab-forming process.

The macrophage. The data indicating that blockade of the reticuloendothelial system (RES) inhibits Ab formation have usually been interpreted to mean that macrophages form Ab (Bieling and Isaac, '21, 22a, b, c; Gay and Clark, '24; Vannucci, '24; Cannon *et al.*, '29). If, however, the function of the macrophage is mainly to localize and partially digest particulate Ag, one would expect RES blockade to inhibit Ab formation to soluble Ag to a lesser degree. To our knowledge, reticuloendothelial blockade has never been shown to depress antibody production to a soluble antigen (Standenath, '23; Gay and Clark, '24; Jungeblut and Berlot, '26).

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[3] In this paper, the term "primitive reticular cell" is used to designate a cell with pale, oval nucleus and scanty, poorly defined cytoplasm. It is not an active phagocyte.

More-direct evidence was presented by Sabin ('39) and Hartley ('40) that macrophages produce Ab. Sabin's evidence was based on the shedding of cytoplasmic material from cells that had engulfed an insoluble azoprotein antigen. There is, however, no definite evidence that the desquamated material she described was antibody. Her experiments were complicated by the use of multiple injections of the labeled antigen. Hence the cellular phenomena observed are difficult to correlate with the time of antigen injection. If macrophages digest particulate Ag and then transfer a soluble component to another cell for Ab fabrication, the time of "shedding" in relation to Ag injection is critical. When multiple Ag injections are given, the shed material may represent either Ab or processed Ag.

Hartley's conclusions are compatible with the premise that macrophages form Ab. He observed that macrophages represent the main cell type in an aluminum hydroxide tissue depot (Hektoen and Welker, '33). After Ag injection into this alum granuloma, Ab was apparent there before it was found in the serum; however, the histologic reaction in the depot after this local Ag injection was not described. Hartley also noted other types of cells in the depot, among them lymphocytes. It is therefore possible that cells other than macrophages formed the Ab. Later attempts to isolate concentrated antibody from macrophage exudates were unsuccessful (Ehrich *et al.*, '46).

The lymphocyte. The evidence supporting the concept that lymphocytes fabricate Ab is no more convincing than that supporting the macrophage as the Ab-forming cell. A masterful review of the published data, however, indicates that lymphocytes somehow share in the Ab-forming process (McMaster, '53). Much of the work used to support the concept that small lymphocytes synthesize Ab (Ehrich and Harris, '42; Dougherty, Chase, and White, '44; Dougherty, White, and Chase, '44; Dougherty *et al.*, '45; Chase *et al.*, '46) lacks consistent confirmation (Eisen *et al.*, '47; Craddock and Lawrence, '48; Fischel *et al.,* '49; Germuth and Ottinger, '50; Malkiel and Hargis, '52; Harris and Harris, '56).

Several observations suggest that the lymphocyte contains Ab. Ehrich and Harris ('42) and Harris *et al.* ('45) noted consistently that the number of small lymphocytes in the efferent lymph increased at a time that would indicate a relation to the immune response. Extracts of these cells contained more Ab than the surrounding lymph. Moreover, Wesslén ('52), using *in vitro* tissue incubation, demonstrated formation of Ab by a mixture of large and small lymphocytes from the thoracic duct lymph. Even more-direct evidence that antibody is in intimate association with small lymphocytes is given by the studies of Hayes and Dougherty ('54), who demonstrated agglutination of bacteria on the surface of small lymphocytes in tissue spreads from immunized animals. However, the presence of Ab within a particular cell type does not necessarily mean that it was formed by that cell. Among other things, it may mean that the cell is derived from the Ab-forming cell, an interpretation that may apply to the small lymphocyte.

The plasma cell. Attention was focused on the importance of the plasma cell in the immune mechanism by the early observation that these cells accumulated in human tissues during chronic infection (Huebschmann, '13; Bing and Plum, '37). Studies in hyperimmunized animals revealed a similar increase in mature plasma cells in various tissues (Bjørneboe and Gormsen, '43; Fagraeus, '48). This evidence, along with the observation that agammaglobulinemia in man is correlated with a decrease or absence of tissue plasma cells (Good, '54, '55) and the discovery of Ab in plasma cells by utilization of fluorescein-labeled Ag (or Ab) (Coons *et al.,* '55; Leduc *et al.,* '55; White *et al.,* '55) support the concept that mature plasma cells are related to Ab formation and may store Ab. The scarcity of mature plasma cells in the lymph node or spleen during or after the response to a single injection of Ag in the rabbit (Harris and Harris, '49) or the rat (La Via *et al.,* '57) suggests that these cells may not be active in Ab formation under these conditions. In these studies, very definite cell changes, not involving plasma cells, were observed after an initial Ag injection.

The intense pyroninophilia that mature plasma cells exhibit is related to an increase in cytoplasmic ribonucleic acid (RNA) (Brachet, '40), and usually correlates with protein synthesis (Caspersson, '50; Brachet, '52). It must be emphasized, however, that there are probably many pyroninophilic cells in the spleen and lymph nodes besides those related to Ab formation and that the mere presence of pyroninophilia does not constitute evidence of Ab formation nor is it specific for plasma cells. Pertinent to the question of whether mature plasma cells form Ab is the fact that most if not all reports that relate an increase in mature plasma cells to this phenomenon have been based on investigations on animals receiving multiple injections of Ag or prolonged stimulation from adjuvant-antigen combinations. The observations summarized later in this paper indicate that the transformation to the mature plasma cell is but one of the possible end stages of the Ab-forming cell.

Other cell types. Some investigators who have studied the spleen or lymph node during Ab formation apparently concluded that the cell that forms Ab should be called a lymphoblast or an immature plasma cell. Others have been unwilling to commit themselves to this extent and have referred to these proliferating cells as splenic tumor cells or transitional cells. We shall refer to the large pyroninophilic cells, which seem to be active in Ab production, as "Ab-forming cells."

EXPERIMENTAL APPROACH

Procedure. Groups of young adult male albino rats (200–250 g) were injected intravenously with a single dose of Ag. *Salmonella typhi* (formalized vaccine), sheep erythrocytes or their stromata, and heat-killed Friedlander's bacilli were given for antigenic stimulation. The methods used for Ag preparation, Ab determination, Ag labeling, and other factors have been described by Wissler *et al.* ('53), Fitch *et al.* ('53, '56), and La Via *et al.* ('56). The injected rats were killed at intervals, suitable tissues were taken for histologic examination, and serum was obtained for Ab determination.

The data have resulted from a circumscribed study of the cellular aspects of Ab formation in the spleen of the rat after a single intravenous injection of a particulate Ag. These conditions were chosen because previous work had shown that nearly all the Ab formed in the rat under these circumstances was formed in the spleen (Rowley, '50; Wissler *et al.,* '53). This is in contrast to the reaction of the rabbit to similar stimulation, where Ab formation appears to be more diffuse (Taliaferro and Taliaferro, '50, '56; Jacobson *et al.,* '50; Jacobson and Robson, '52). Support for this approach was furnished by the fact that in the rat only the spleen showed morphologic changes that could be correlated with Ab formation (Fitch *et al.,* '53). Early in these studies it was apparent that most of the Ag localizing in the spleen was present in the red pulp (Fitch *et al.,* '53; La Via *et al.,* '56).

Cellular sequence in the rat spleen. After a latent period (48 hours), definite cellular proliferation was evident in the red pulp. The cells that first proliferated were characterized by large vesicular nuclei and pale irregular cytoplasm with little

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Fig. 1A Early stages of cellular proliferation in the red pulp of the rat spleen 48 hours after a single intravenous injection of a standard quantity of *S. typhi* vaccine. The mitotic activity and the nature of the large cells that have not yet developed much pyroninophilic affinity are illustrated under oil immersion (methyl green pyronine).

B Cell population in the red pulp of the spleen at the peak of cell proliferation 4 days after a single standard intravenous injection of the *S. typhi* vaccine. The oil immersion photograph demonstrates the antibody-forming cells with abundant pyronine-positive cytoplasm, large, sometimes eccentric nuclei, and characteristic chromatin pattern (methyl green pyronine).

C Characteristic cellular pattern in the splenic red pulp 6 days after a single intravenous injection of the *S. typhi* vaccine showing the marked decrease in pyroninophilic cells and the appearance of numerous small dark cells with sparce cytoplasm, presumably small lymphocytes (methyl green pyronine).

D Eight days after intravenous *S. typhi* vaccine, the splenic red pulp has returned to its preinjection appearance except for a few additional mature plasma cells, most of which have a moderate quantity of pyronine-positive cytoplasm. No evidence of nuclear debris is seen in the histiocytes at this time, nor was any seen earlier, to suggest that the small dark cells noted at 6 days have undergone necrosis (methyl green pyronine).

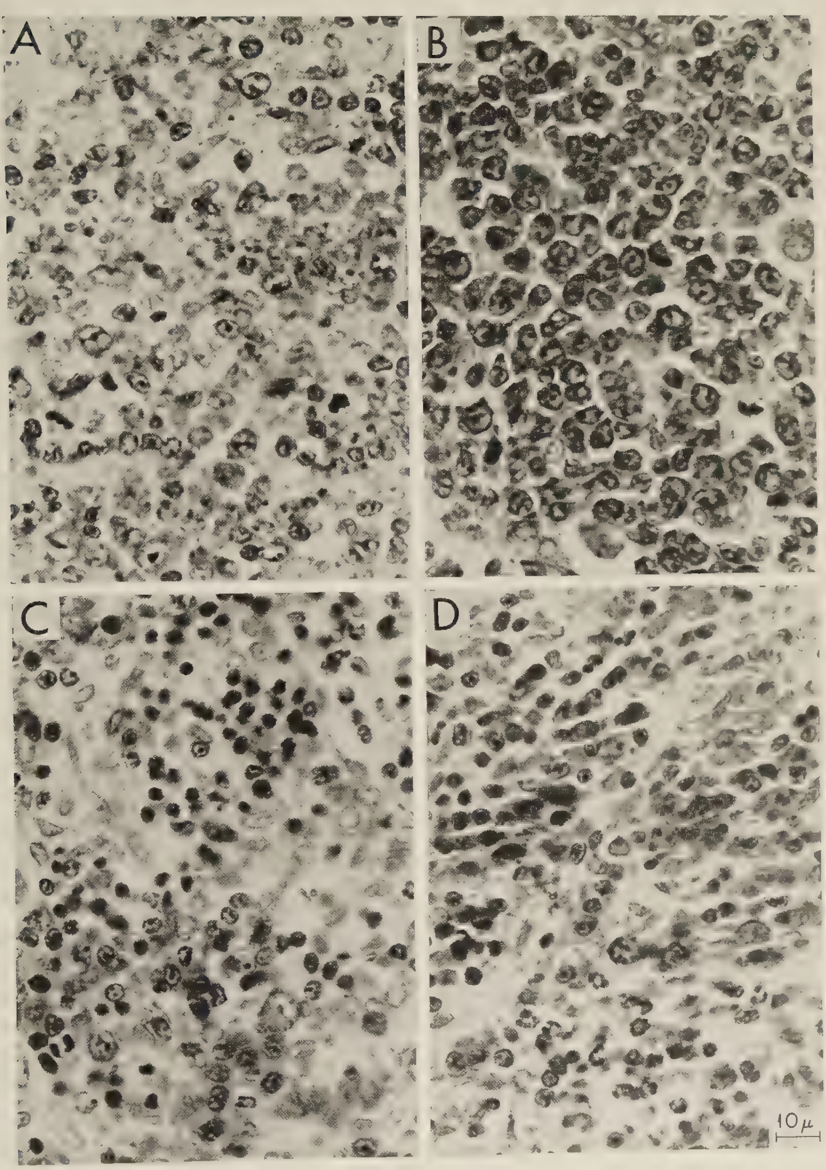

Figure 1

evidence of pyroninophilia. At this time and for 12 to 24 hours thereafter, these cells with evident cytoplasmic processes resembled closely the primitive reticular cells of the splenic red pulp (fig. 1A). About 48 hours after Ag injection, the mitotic rate of these Ab-forming cells increased. The cytoplasm became steadily more pyroninophilic with more regular cell borders, and the nucleus seemed slightly smaller and richer in chromatin. Sometime before 96 hours, the mitotic activity seemed to decline and the red pulp of the spleen presented a fairly uniform population composed of large cells with coarsely granular, intensely pyroninophilic cytoplasm and with nuclei that showed marginal chromatin and prominent nucleoli (fig. 1B). These cells, which do not resemble mature plasma cells, are probably the principal Ab-forming cell type.

By 6 or 7 days after Ag injection, most of these pyroninophilic cells had disappeared from the splenic red pulp. There was no evidence of cell death, and great numbers of small darkly stained cells with scant cytoplasm made a transient appearance (fig. 1C). It seems likely that most of the Ab-forming cells shed their cytoplasm 4–6 days after injection and that the small ''dark'' cells were derived from the Ab-forming cells by direct transformation. The swift disappearance of these small cells from the spleen suggested that they were liberated into the blood stream after the peak of intracellular Ab formation, about 5–7 days after Ag injection. Closely spaced observations indicated that the pyroninophilic material in the Ab-forming cells decreased before the number of cells in the red pulp of the spleen decreased. Both cell number and spleen weight declined rapidly after this decrease in pyroninophilic material. Thus, by 8 days after injection of particulate Ag, the appearance of the spleen was relatively normal with the possible exception of a slight increase in the number of mature plasma cells in the red pulp (fig. 1D).

Correlation of other findings with the cellular changes. It is apparent in figure 2 that there is a definite relation between the increase and subsequent decrease of pyroninophilic cells in the red pulp of the spleen and the rise and fall of circulating

Ab concentration after Ag injection. After an initial 24–48 hour induction period, there is an increase in pyroninophilic cells that precedes by about 2 days the initial rise in circulating Ab titer. This lag period is apparent throughout the experimental period. It suggests that the intracellular process related to Ab fabrication precedes by about 2 days the liberation of Ab into the blood stream.

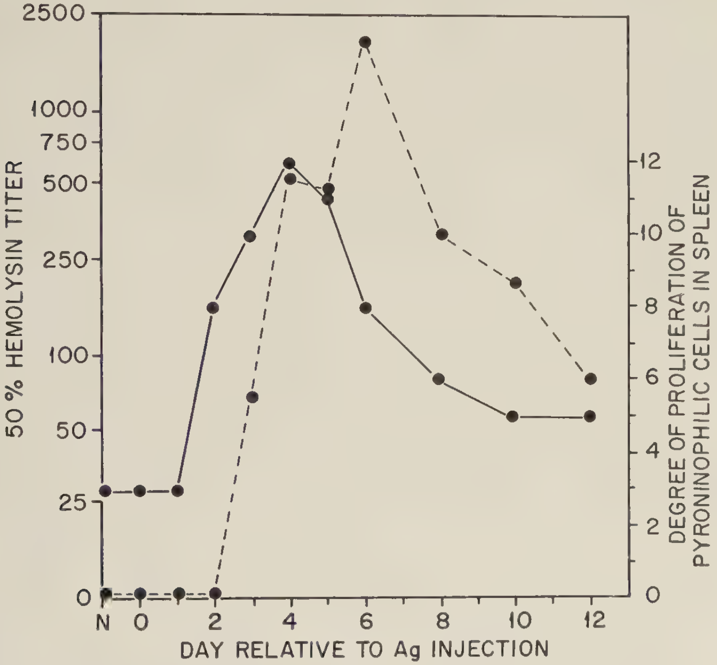

Fig. 2 Serum hemolysin concentrations and evaluation of splenic red pulp pyroninophilic cell population showing a definite correlation of these two responses at varying times after a single intravenous injection of washed sheep erythrocytes mixed with typhoid vaccine. Each point represents the average of three observations.

Study of the mitotic activity in the red pulp of the spleen supports the idea that the Ab-forming cells are newly proliferated cells. Figure 3 indicates that a rise in the mitotic rate precedes the increase of pyroninophilic cells by at least 24 hours. It appears that these cells first divide and then become increasingly pyroninophilic; furthermore, the changes in

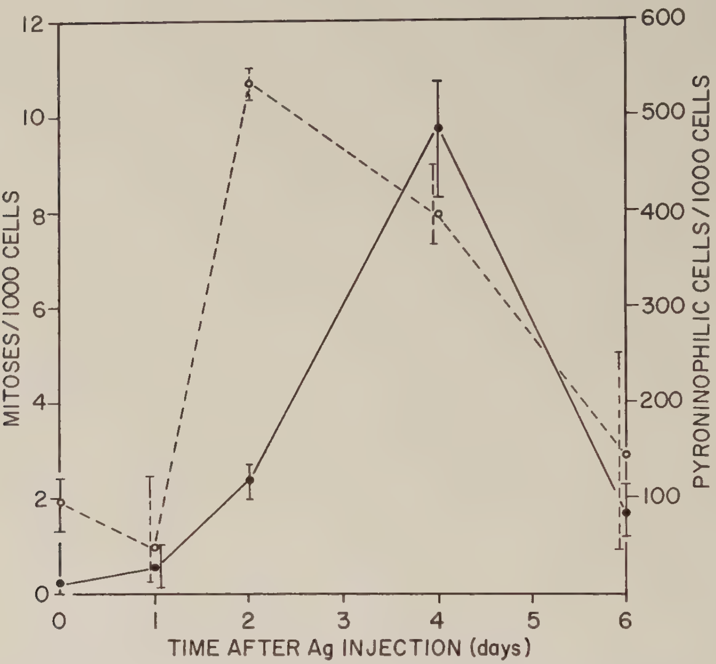

Fig. 3 The mitotic activity is related to splenic red pulp pyroninophilia by means of actual cell counts of random fields in the splenic red pulp. Note the early rise in mitotic index (2 days) as compared to the later increase in numbers of deeply pyronine-staining cells. Each point represents the average of three determinations.

spleen weight indicate a real increase in the protoplasm, which reaches its peak 4 days after injection of most antigens. Although in some experiments the early spleen weight changes were complicated by a rise that was probably a reaction to bacterial endotoxins, the weights after 48 hours correlated with the histologic evidence of cell proliferation. This was particularly evident in response to relatively nontoxic antigenic stimulation, such as sheep red blood cells (fig. 4). Further studies of the factors contributing to the increased splenic weight at various times after Ag injection show that the water and hemoglobin concentrations of the spleen remain relatively constant although the concentration of nitrogen increases (C. H. Gunderson, unpublished observations).

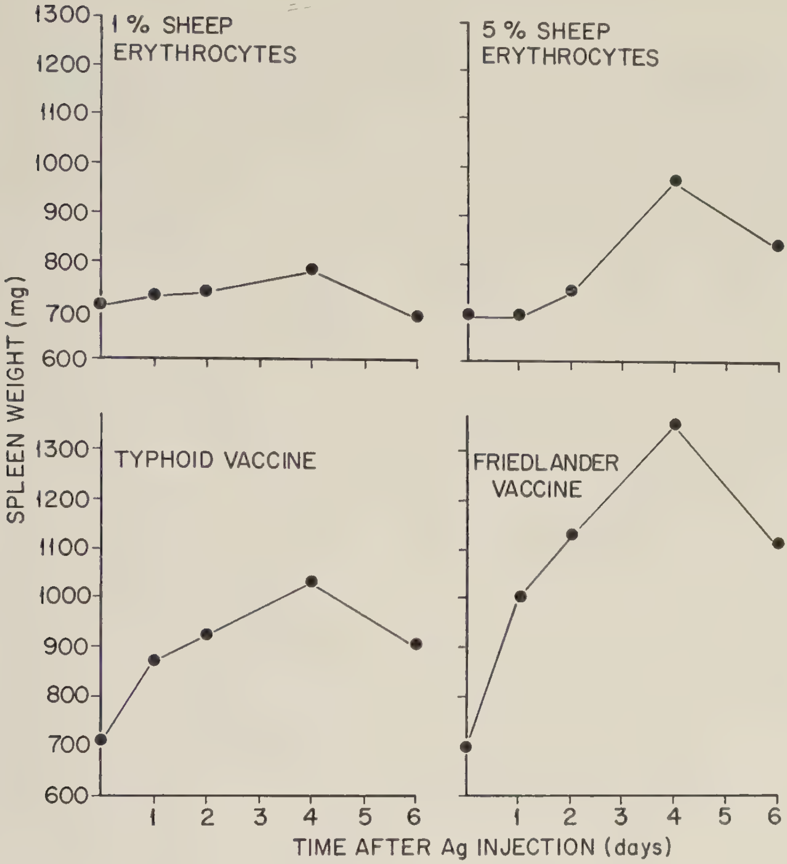

Fig. 4 Average total spleen weights at varying intervals after the intravenous injection of the indicated particulate antigenic material. In most instances, each point is the average of three spleens. Note the peak at 4 days in all cases and the difference in response with the different antigenic materials.

Support for the concept that the appearance of the large pyroninophilic cells is related to Ab-protein synthesis is furnished by tissue culture data derived from spleens removed on the second day after Ag injection (M. F. La Via, unpublished observations). Results given in table 1 indicate definite incorporation of C^{14}-glycine into specific typhoid Ab in spleen explants from Ag-injected rats incubated for 48 hours. Fur-

TABLE 1

Incorporation of C^{14}-glycine into Salmonella typhi agglutinin in vitro by minced rat spleen

TIME AFTER ANTIGEN INJECTION	LENGTH OF INCUBATION	RADIOACTIVITY IN TOTAL MATERIAL BOUND TO ANTIGEN	RATIO OF EXPERIMENTAL OVER CONTROL	AGGLUTININ TITER	
				In serum	In incubation medium
hr	*hr*	*cts/min*			
0	48	39	. .	0	0
48	48	117	3.0	0	1:20
48	48	159	4.0	0	1:20
0	72	74	. .	0	0
48	72	248	3.3	1:2	1:20
48	72	262	3.5	1:4	1:40

thermore, histologic study of these explants at the beginning and end of the *in vitro* incubation period revealed an increase in pyroninophilia of many of the cultured cells (fig. 5A and 5B).

Evidence that the Ab-forming cells are necessary for Ab synthesis is furnished by 4 published studies from this laboratory. It was shown that X irradiation of the entire body, severe protein depletion, or acute administration of an essential amino acid antagonist depresses or abolishes Ab forma-

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Fig. 5A, B Minced splenic fragment (A), incubated 48 hours in a medium containing C^{14}-glycine. The spleen was removed from a rat 48 hours after intravenous antigen injection. Note the abundant intensely pyroninophilic cells as compared to a similarly incubated spleen (B) from a noninjected control rat (methyl green pyronine).

C Splenic red pulp of a rat killed 2 days after a mixture of washed india ink particles and *S. typhi* vaccine was given intravenously. There is no evidence of the carbon in any of the newly formed red pulp cells that have proliferated in response to the antigenic material (methyl green pyronine).

D When the washed india ink was given one hour before the animal was killed and when death was at varying intervals after a single intravenous injection with *S. typhi* vaccine (in this instance the interval was 4 days), there was no evidence of phagocytic activity by the antibody-forming cells (methyl green pyrinine).

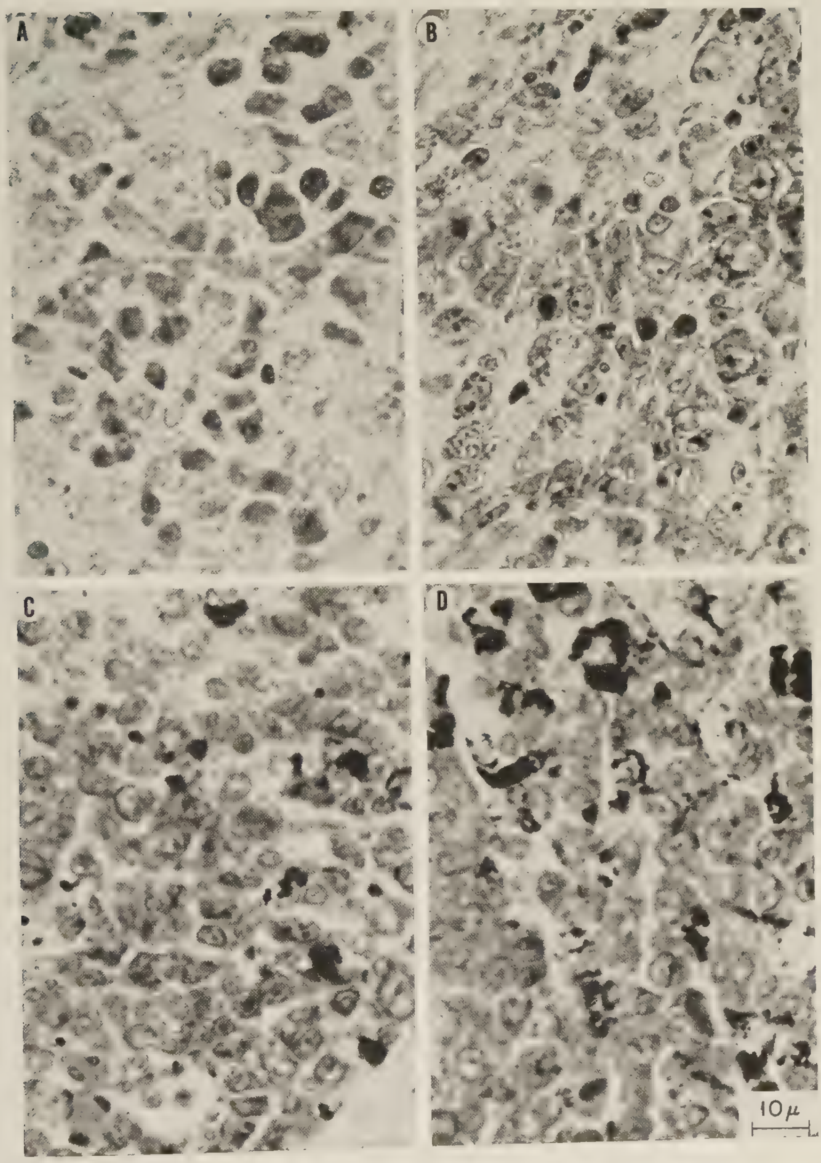

Figure 5

tion, and each treatment leads to a marked inhibition of the proliferation of the Ab-forming cells. X irradiation of the total body interferes with antibody formation but gives little evidence of damage to the phagocytic or digestive properties of the splenic macrophages (Fitch *et al.*, '53). The amino acid inhibitor depresses antibody formation without evidence of any change in the splenic lymphoid tissue (Wissler *et al.*, '56). Neither protein deficiency nor amino acid antagonist has any appreciable effect on the mature plasma cell (La Via *et al.*, '56; Wissler *et al.*, '56). Under many experimental conditions involving varying chronologic relations between Ag injection and irradiation, the depression of cellular proliferation and the subsequent decrease in the appearance of pyroninophilic cells in the splenic red pulp of the spleen have been correlated with the lack of formation of circulating Ab (Fitch *et al.*, '56).

The cellular pattern and its problems. A summary of the cellular transformations occurring in the rat spleen during Ab formation and the major questions posed is presented in figure 6. This is similar in some respects to diagrams published by others (McNeil, '50; Ehrich, '55). Here, the particular problems that seem to need most active investigation are indicated. How the Ab-forming cells receive an Ag stim-

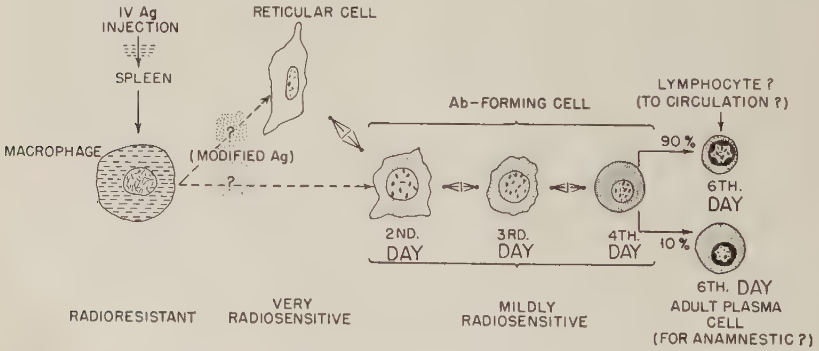

Fig. 6 A summary of a dynamic cellular concept of antibody formation based on observations in the rat spleen after "primary" antigenic stimulation given intravenously. The question marks indicate the areas where more study seems to be especially needed.

ulus is puzzling. Most of the particulate Ag that reaches the spleen appears to localize in a definite pattern in the phagocytic cells of the red pulp (Fitch *et al.*, '53; La Via *et al.*, '56). The Ab-forming cell, on the other hand, appears to be derived from nonphagocytic cells. Evidence for this was obtained by injecting intravenously a mixture of washed india ink particles and I^{131}-labeled typhoid bacilli. Study of the radioautographs prepared from the spleens of these rats indicated that the carbon and the I^{131} activity were localized in the same phagocytic cells. Further experiments showed that the carbon did not appear in the proliferating cells of the splenic red pulp, as might be expected if the Ab-forming cells were being derived from phagocytic cells (fig. 5C). Furthermore, these newly proliferated cells did not exhibit any phagocytic capacity when carbon particles were injected shortly before the animals were killed (fig. 5D).

The fate of the Ab-forming cells, which disappear rapidly between the fourth and seventh days after Ag injection, has not been established. Histologic evidence seems to indicate that these cells enter the blood stream as cells resembling small lymphocytes. Several attempts to demonstrate this by means of serial leukocyte counts in normal and spleen-shielded irradiated animals at intervals after Ag injection (C. H. Gunderson, unpublished observation) revealed no rise in circulating mononuclear cells coincidental with the disappearance of Ab-forming cells. It is questionable whether the probable increment in cell number is large enough to be detected by this relatively crude technique. It should be noted that evidence obtained in the rabbit makes it seem likely that increased numbers of cells resembling small lymphocytes are released into the lymphatic circulation at about the time the cellular proliferation reaches its peak in the popliteal lymph node, 3–4 days after a single injection of antigen into the foot pad (Ehrich and Harris, '42). We are now attempting to use tracer methods to detect the release of cells containing newly formed Ab into the circulation of the rat.

How the plasma cell fits into the cellular sequence of Ab formation in the rat is still not clear. There is no doubt that the rat is capable of forming mature plasma cells, but there is some question of whether it develops the same progressive increase in plasma cells after hyperimmunization that other investigators have demonstrated in the rabbit (Bjørneboe and Gormsen, '43; Fagraeus, '48). It is apparent, moreover, that there are several important differences between the immunologic reactions of the rabbit and rat. For example, it has been reported that the rat produces precipitins in only small quantities, if at all, and that it does not develop a tuberculin reaction (Rich, '51), that it develops an Arthus phenomenon only with difficulty (Kenton, '41), that it demonstrates an unusual type of anaphylactic response (Rich, '51), and that it may not show a specific anamnestic reaction. It is difficult to correlate these species differences with differences in the cellular reaction after Ag injection. Preliminary studies in this laboratory suggest that only a small and delayed cellular response occurs in the rat spleen after a large intravenous injection of a soluble Ag. Other observations indicate that far fewer of the Ab-forming cells mature into plasma cells in the rat than the rabbit, and that the rat has a greater splenic cellular response to single intravenous injection of Ag than the rabbit.

DISCUSSION

Relation to previous work. In spite of possible differences among the mammalian species, the cellular pattern that occurs during Ab formation in the rat spleen has a great deal in common with patterns previously observed in the rabbit by others. Using multiple injections of a soluble Ag in the rabbit, Rich *et al.* ('39) studied the *in vitro* and *in vivo* characteristics of the cells that they suggested were active in Ab formation, and called them "acute splenic tumor cells." They believed that these cells were morphologically and kinetically similar to lymphoblasts and had their early origin (24–48 hours) from the cells of the Malpighian corpuscle. As yet there is no convincing evidence in the rat spleen to support the contention that Ab-forming cells are derived from the splenic follicle, but otherwise they resemble those described by Rich and his associates.

The descriptions of the sequential lymph node reaction in the rabbit after a single Ag injection (Ehrich and Harris, '42; Ehrich *et al.*, '49; Harris and Harris, '49) are similar in many ways to the observations in the rat spleen. As mentioned earlier, these workers noted an increase in the number of Ab-containing lymphocytes in the efferent lymph that began at a time when the lymph node hyperplasia became intense (3–4 days after Ag injection). The question arises whether the Ab extracted from these cells in the efferent lymph may have been contained in a large number of small lymphocytes derived from Ab-forming cells in the lymph node or whether it may have come from a small number of the larger cells that were liberated into the lymph during the active process of Ab formation.

The large pyroninophilic cells called Ab-forming cells in this study seem comparable to the cells that Fagraeus ('48) has called "transitional cells" in the secondary response in the rabbit spleen. Similarly, the Ab-containing cells identified by Leduc *et al.* ('55) were similar large immature cells in the medullary areas of the stimulated rabbit lymph node. Using the fluorescein-labeling technique, these investigators followed the cellular aspects of the Ab-forming process sequentially and noted that large cells, seen early after Ag injection, multiplied and differentiated and accumulated increasing quantities of Ab in the cytoplasm until cells developed that resembled mature plasma cells. The primary and secondary responses were very much alike, with identical morphologic cell types being involved. In the secondary response, however, many more Ab-containing cells were produced. It is important to note that Ag was not identified in cells of the plasma cell series. It may have been present, but not demonstrated because it was bound to newly formed Ab or because the quantity was insufficient to be seen by this method. Furthermore, there was no evidence of derivation of the Ab-containing cells from the Ag-localizing cells. Although the number of Ab-containing cells decreased remarkably between the fourth and eight days after a secondary stimulation, Leduc and her associates reported no evidence of transformation of the immature plasma cell into a small lymphocyte-like cell.

Irradiation as a technique for the study of the cellular pattern. The cellular mechanism of Ag catabolism and Ab formation may be studied in the irradiated animal. If total-body irradiation is administered a day or two before Ag injection, the phagocytic function of tissue macrophages is found to be relatively radioresistant, and the digestion of phagocytosed particulate Ag is not impaired in the early postirradiation period (Fitch *et al.*, '53; Ingraham, '55), although it may be depressed later (Donaldson *et al.*, '56). Results indicate that the most radiosensitive portion of the cellular-immune reaction is localized in that part of the process during which the Ag stimulus activates the reticular cell. It may involve an altered stimulus or an altered response by an altered cell (Taliaferro *et al.*, '52; Taliaferro and Taliaferro, '54). There is some evidence that the stimulus is not altered, since in some studies of Ab formation in irradiated animals the temporarily depressed Ab-forming capacity recovers after a prolonged period even though no more Ag is administered (Fitch *et al.*, '56; Silverman and Chin, '56).

Morphologically, the macrophage, the reticular cell, the Ab-forming cell, and the mature plasma cell all appear to be resistant to necrosis caused by radiation exposures that depress Ab formation. On the other hand, mitosis of the Ab-forming cells is radiosensitive, so that multiplication of these cells is depressed temporarily when they are irradiated during the proliferative period (Fitch *et al.*, '56). In general, irradiation after antigen injection alters Ab formation by lengthening the induction period and by slowing the rate at which Ab appears in the blood. Under some conditions, it also seems to reduce the maximum Ab concentration achieved.

These effects can be understood at least partially, if they are correlated with the effects of irradiation on the cellular response (Fitch *et al.*, '56). Several characteristics of the altered immunologic mechanism of the irradiated animal are still poorly understood. These include the enhanced formation of Ab when the antigen is given 1–4 hours before irradiation (Taliaferro and Taliaferro, '56), the radioresistance of the anamnestic response under some conditions (Dixon *et al.*, '52) and its radiosensitivity under others (Taliaferro *et al.*, '52; Talmage *et al.*, '56), and the more rapid recovery of the immunologic reaction to some Ag than to others (Hale and Stoner, '54). Perhaps study of the histologic response in relation to these phenomena may help to clarify the mechanisms involved.

Cell transfer as an investigative technique. The transfer of lymphoid cells to normal or previously irradiated recipients is another approach that may aid identification of the Ab-forming cells. The transfer of cutaneous tuberculin hypersensitivity to normal animals by homologous lymph node cells from sensitized animals has been repeatedly demonstrated (Landsteiner and Chase '42; Chase '45; Kirchheimer and Weiser, '47; Lawrence, 49, '56). There has been increasing interest in the use of this technique for study of the various phases of the Ab-forming mechanism (Harris and Harris, '55; T. N. Harris *et al.*, '56; S. Harris *et al.*, '56; Oakley *et al.*, '54). It is difficult to evaluate the contribution of many of these studies to an understanding of the cellular mechanism of Ab formation since the transfer site has rarely been examined histologically.

Roberts *et al.* ('57) have reported a study of the cellular transformations occurring after transfer of cells from previously stimulated rabbits into X-irradiated rabbits. On the basis of their observations, they question the importance of cellular proliferation in Ab formation but recognize that this portion of the cellular pattern of the Ab response may be modified after transplantation. Similar lack of mitotic activity appears to occur when Ab is formed in tissue culture

(Fagraeus, '48; M. F. La Via, unpublished observations). Perhaps the most challenging aspect of the morphologic study of Roberts *et al.* ('57) is the implication that under some conditions, the Ab-forming cells originated from phagocytes. It must be emphasized that in this study the donor cells were obtained from hyperimmunized animals. Further study will be necessary to ascertain whether phagocytic cells from such animals have different potentialities than those from animals not previously immunized. The well-established capacity of the circulating lymphocytes to develop into phagocytic macrophages (Maximow and Bloom, '52) and the implication from studies in this (La Via *et al.*, '57) and other (Harris and Harris, '49) laboratories that some of the Ab-forming cells develop into small lymphocytes suggest that macrophages may be produced, after multiple antigenic stimuli, that may respond in an altered fashion to subsequent contact with the same Ag.

Unfortunately, the mixtures of cell types involved in the cell transfer experiments that have been reported and the frequent use of cells harvested after multiple antigenic stimulation have made it difficult to interpret the results in relation to any morphologic pattern of Ab formation. Further work with this technique but with more attention to these variables and to the histologic aspects may yield valuable information about the cellular mechanism of Ab formation.

Much attention has been given in discussions of the kinetics of Ab formation to the self-replication of some type of unit that might explain the relatively greater strength of the secondary response, the exponential rise in humoral Ab concentration under some immunologic situations, and the continued production of Ab over long periods. These studies and observations discussed previously suggest that the self-replicating unit may be a cell rather than an intracellular enzyme system. One would, then, visualize that the Ab-forming cell, originally arising by rapid proliferation and differentiation, retains some altered capacity even though it is later morphologically modified and widely distributed as a lymphocyte (perhaps maturing to a macrophage) or as a plasma cell. On reinjection of similar antigen, some of these cells with previous exposure to this Ag may have a considerably greater potential for multiplication and Ab formation than before. This concept would seem to be compatible with the frequent observation that the rabbit shows much greater cellular proliferation after a second injection of Ag than after the first (Fagraeus, '48). Furthermore, Leduc *et al.* ('55) showed that many more cells appear to contain Ab after a second injection than after the first.

Study of the early cellular changes in the specific anamnestic response may indicate how previously modified cells augment and accelerate the formation of Ab.

These problems emphasize the need for further consideration of the process of Ab formation in relation to a dynamic concept of cellular response, an approach that should go hand in hand with increasing investigation of the immunochemical processes involved.

SUMMARY

The exclusive role of the macrophage, the lymphocyte, or the plasma cell in the Ab-forming process is not supported by existing evidence. Observations in the rat spleen after a single primary intravenous injection of particulate Ag material have revealed a cellular pattern that involved proliferation, differentiation, and mobilization of cells in the red pulp. Although the macrophage (histiocyte), small lymphocyte, and mature plasma cell are each involved, the principal Ab-forming cell appears to be a large, pyroninophilic cell derived by active mitosis from a primitive fixed reticular cell whose phagocytic powers seem to be limited, if they exist at all. After most of the Ab to this primary stimulus has been formed, this cell type apparently transforms into either a small lymphocyte or a mature plasma cell. In the rat spleen, under the conditions studied, the predominant pathway is to small lymphocytes. These small cells then disappear from the splenic red pulp, with no evidence of necrosis, and they

seem to enter the circulation. X irradiation of the total body, prolonged dietary protein deficiency, or the acute administration of an Ab-inhibiting amino acid antagonist each depresses the immune response and each interferes with proliferation and differentiation of the Ab-forming cell. Many auxiliary observations support this cell type as the principal source of Ab synthesis. Further study should indicate how this cell receives the Ag stimulus and clarify the manner in which it so rapidly disappears from the spleen when Ab formation is almost completed. Chemical and cellular aspects of Ab synthesis should be better correlated.

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OPEN DISCUSSION

HEIDELBERGER[4]: I should like to take advantage of my position as Chairman by opening the discussion with a plea for one or two immunochemical considerations; and I should like to ask Dr. Wissler whether he has taken these entirely into account. It greatly complicates any interpretation of results such as these to find that Dr. Wissler injected what he calls an Ag but what we all know is really a vast collection of Ag, and obtained a very complicated cell response in which he can distinguish between six different varieties of cells. Then by measuring a single Ab, he attempts to correlate the response of six different cells with the Ab response to perhaps only one out of twenty or ten or n Ag injected. This has complicated matters a great deal for me, an immunochemist. It may be entirely justified, but it makes me wonder.

It also seems to me that studies such as this ought to be correlated with physical-chemical and organic chemical studies of the kind of Ab produced, because we know that different Ag injected by different routes, in some animals at least, and sometimes different Ag injected by the same route will produce Ab with totally different immunological properties. Such Ab may appear in different serum fractions, and therefore are not necessarily produced by the same cells or at the same time.

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[4] M. Heidelberger, Rutgers University.

I have wondered why this kind of study, such as Dr. Wissler made in the rat, has not been made in the horse, about which we have a great deal of immunochemical information. One could probably anesthetize a horse and make biopsies at suitable periods; it might not be too easy, but if one really wanted to do it one could. We know, for instance, that, taking what for the moment may pass as a single Ag, if we inject diphtheria toxoid or a foreign serum albumin into a horse, we get antibodies that are usually in the water-soluble fraction of the globulin and that show a typical toxin-antitoxin response; we get these antibodies if we inject the Ag subcutaneously.

On the other hand, in the same animal, if we inject a globulin or a bacterial Ag or perhaps red cells — I don't know — or collections of antigens of this sort, we get a curve that is exactly like the ordinary precipitin curve obtained in rabbits. The antibodies are mainly in the water-insoluble fractions and have a molecular weight five or six times that of the Ab in the water-soluble fractions and that give rise to the toxin-antitoxin type of curve.

It seems to me then that by our choice of animal we could in some instances greatly simplify the problem, and by our choice of something approaching a single Ag in an animal in which the Ab response had been changed in some of the overall features, we might simplify the interpretation.

WISSLER: I agree that there is a lot of room for improvement. As a pathologist, I am interested in infection so it is natural for me to study the cellular reaction to a bacterial vaccine in order to demonstrate the essential features of the process. I think this system has some advantages over some that have been used. The antigens are localized very quickly in a tissue that apparently has an important role in Ab formation, so that the timing of the reaction is easier to follow. This type of approach needs to be extended to pure antigens and comparisons need to be made between different types of antigens.

I agree that some part of the secret of why some antibodies are found in one part of the globulin spectrum while others are found in other parts may be related to the degree of differentiation of the cell at the time that the antibodies are released. Dr. Frank Putnam and I have tried to correlate the types of globulin released in multiple myeloma with the types of cells that we saw in the neoplasm. Results obtained with the few patients we have studied suggest that there might be a definite correlation of the numbers of cell types seen and the number of abnormal globulins being made.

Both of Dr. Heidelberger's suggestions are well taken, and I hope that other people who are better equipped than we to work with some of these Ag will be studying the cellular reaction in Ab synthesis and that we can go ahead and study some purer systems.

WOLFE [5]: Dr. Makinodan, Dr. Ruth, and I published two papers in the Journal of Immunology in early 1954. We injected chickens with crystalline bovine serum albumin and studied the cell types during Ab formation, and we also made RNA, DNA, and Ab-N determinations. The cell types, I believe, were quite similar to those described by Dr. Wissler.

TALIAFERRO [6]: I should like to point out that much the same sequence of cellular changes that Dr. Wissler describes takes place during the development of immunity to malaria. My interpretation of the changes, however, differs somewhat. For example, the spleen contains many large, fixed reticular cells that, when they become phagocytic, cannot be distinguished from a phagocytic macrophage. This large, fixed reticular cell can also develop into a large free basophilic cell that I include in the large lymphocytes. This free cell has wide mesenchymal potencies, including the ability to transform into lymphocytes of various sizes, macrophages, and plasma cells. Its variable appearance probably explains why some investigators do not accept it as an entity but divide it into several cell types and give it a variety of names.

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[5] H. R. Wolfe, University of Wisconsin.

[6] W. H. Taliaferro, University of Chicago.

When we follow the localization of particulate Ag, we may not be following the fraction of the Ag that stimulates Ab formation. Most of a particulate Ag is phagocytosed by macrophages, and its digestion by them may prevent it from taking part in Ab formation. Most workers agree that a very small part of the Ag is functional in Ab formation. It is interesting, therefore, that we have found that the large basophilic lymphocyte in the chicken is occasionally phagocytic to the extent that it ingests red cells infected with malaria. Possibly, phagocytosis by these cells, small as it is, represents the fraction of Ag that is functional. Such a possibility, if true, would eliminate one difficulty in most theories of Ab formation, viz., the antigenic stimulus would not have to pass from the macrophage to the lymphoid cell that is supposed to produce Ab.

Finally, you mentioned the difference between Dr. Dixon's finding that the anamnestic response is radioresistant and our finding that the hemolytic anamnestic response is radiosensitive. The work of Talmage and Thomson indicates that the hemolysin response can be made resistant if sufficient Ag is used.

Coons[7]: Dr. Wissler's beautiful pictures and his comments have raised questions, some of which were directed to me. Dr. Leduc and I were not able completely to bridge the gap between the reticular cell and the first cell in which we could demonstrate Ab in the rabbit. However, there seems to be a general opinion among students of these cells that these large cells do spring from reticular cells.

[7] A. H. Coons, Harvard Medical School.

Once this large primitive-looking cell, which looks like a primitive leukocyte or hemocytoblast, can be shown to contain antitoxin, the course of events in its development is exactly the same in Dr. Wissler's material in the rat as it is in the material that others have studied in the chicken and in our rabbit material, except that in the rabbit after one injection of diphtheria toxoid, there are very few cells and very little cellular activity in the lymph node. If we could not stain for antitoxin, we could not really demonstrate anything in these popliteal lymph nodes. Dr. Wissler's pictures of the rat are very similar to the pictures that we see in the rabbit lymph node after the secondary injection.

This raises the question of whether this response in the rat is a primary response or perhaps a secondary response, since the rat is Forssman negative and in encountering Forssman material might have become sensitized to the Forssman Ag in the sheep red cells that Dr. Wissler injected. For example, the rat may previously have come into contact with typhoid bacilli and Friedlander's bacillus, which contain antigens that are rather common in families of bacteria. I do not think this question necessarily applies to the chickens that Dr. Wolfe and his colleagues described. As far as we know, bovine serum albumin is not commonly in the environment of chickens, and to this chickens get a remarkable primary response that is like the primary response Dr. Wissler described in the rat.

The question about the fate of the plasma cell at the end of the response—in our material we do not know what happens to these plasma cells. They gradually disappear and a few weeks later, in the primary animal, none can be found. In the secondary animal making small amounts of Ab, months later it is necessary to cut serial sections to find an occasional cell. These cells, however, have the same morphological appearance under the fluorescent microscope as do plasma cells in a more vigorous response. That is about all we can say about them, because they are so rare that we cannot spot them in preparations stained by conventional methods.

Finally, the teamwork between the macrophage and the Ab-producing cell in particulate antigens is, I think, a very likely possibility. If I remember correctly, Ranvier described the macrophage in 1904 and named it the clasmatocyte because it continuously breaks off little pieces of cytoplasm. We have seen this in Kupffer cells containing pneumococcal polysaccharide, where in the lumen of vessels we can sometimes see little pieces of antigenic material that look as though they could be part of the cytoplasm of the Kupffer cells. A particulate Ag coming to rest in a macrophage could therefore be solubilized there and some of it could escape to be picked up as a soluble material.

HEIDELBERGER: That would be somewhat along the line of Dr. Florence Sabin's idea in which she showed that the exfoliation, if one can call it that, of cytoplasm takes place after ingestion of antigen.

COONS: This seems to be a general property of macrophage, whether or not it has an Ag in it.

WISSLER: I would like to thank Dr. Coons for bringing out the points he did. I think the possibility, which he suggests, that we may be studying a secondary response in the rat is certainly one that has to be considered. If that is true, then there must be a more-fundamental difference between the rat and the rabbit than we have appreciated, because the rabbit, during a classical secondary response, apparently matures more of its Ab-forming cells into plasma cells than the rat does, although we know that the rat can make mature plasma cells.

This is really part of the same problem that Dr. Heidelberger raised, and for our work in the future we will have to cast about for antigens to which both the rat and rabbit will respond well. We would like to see if we can uncover any fundamental biological differences that might explain some of these interesting dissimilarities between the immunological reactions of certain species.

Dr. Taliaferro's comments are also very helpful, it seems to me. We have wondered if the primitive reticular cell might not be taking up material that is antigenic either by transfer from the phagocyte or primarily. We cannot answer this question under the rather crude conditions of radioautography that we have used so far. I think this is an area where much better techniques are needed, and I suspect that there are a number of people at this meeting who can contribute methods that will make it possible to follow the fate of antigenic groups at the cellular level — not necessarily the entire antigen, but antigenic groups. This I think would help almost as much as any single improvement in technique to resolve some of the problems of the early changes that are occurring in the Ag and in the cells.

I, for one, think that the primitive reticular cell does take part in phagocytosis, perhaps enough to explain the entire reaction, particularly with soluble antigens. I believe that what Dr. Coons has been calling the reticular cell is the same that we are calling the reticular cell and that he has seen evidence of some antigenic material in these cells when he has given soluble antigens.

HEIDELBERGER: I should like to add just one word about the difficulty of determining whether an animal has had prior experience with an Ag, a supposedly normal animal. I am thinking in particular of Grabar's finding that normal horse sera give precipitates with glycogen. In going over a number of supposedly normal horse sera, we found only one that showed as much precipitate as Grabar obtained — about 20 μg of apparent Ab to glycogen per ml. This was a ''normal horse serum'' being distributed by the New York City Department of Health, who presumably had vital statistics on this horse that led them to believe the serum to be a normal horse serum. I was a little suspicious and tested the serum with types II and XII pneumococcus polysaccharide because I knew that antisera to these types of pneumococcus precipitated glycogen. Both pneumococcal polysaccharides precipitated measurable amounts of Ab, amounts from about 5 μg of nitrogen per ml up to about twelve with these two polysaccharides. Now, depending on one's definition or one's previous knowledge of this horse, one could say that its serum was either "normal" or a type II or XII antipneumococcus serum. It would meet any of those three definitions, depending on what criteria we used.

PAPPENHEIMER[8]: I am just wondering whether you did any mouse protection tests.

HEIDELBERGER: No, we did not, but I still have some of the serum if you would like to do them.

NOVELLI[9]: Dr. Wissler, you discussed the fact that β, 3-thienylalanine prohibited antibody formation and you referred to an experiment of Novick's on adaptive enzyme formation to support the argument that the anolog was acting in some manner to interfere with the concentrating mechanism. I am not aware of Novick's experiments but, in general, most people agree that these amino acid analogs inhibit protein synthesis. The mechanism by which analogs inhibit protein synthesis is not yet clear, but some recent observations of E. W. Davie, V. V. Koningsberger, and F. Lipmann bear on this point. With the purified tryptophan-activating enzyme isolated from beef pancreas, they found that tryptozan (an analog of tryptophan that is known to inhibit growth of microorganisms) was also activated by their enzyme. They suggested that the way amino acid analogs act is to become activated and to be incorporated into protein.

Although this does not seem to be a very important aspect of your theory, I think it would be a much simpler view to visualize the analog as inhibiting protein synthesis, thereby inhibiting proliferation, and thereby inhibiting Ab synthesis.

WISSLER: I did not mean to use this reference as part of the argument as to where the β, 3-thienylalanine acts in our experiment. I think it is interesting that in the experiment that Dr. Novick and Dr. Weiner did, they found evidence of interference with the induction of their enzyme system and no interference with the synthesis of the enzyme.

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[8] A. M. Pappenheimer, New York University College of Medicine.

[9] G. D. Novelli, Oak Ridge National Laboratory.

La Via[10]: Dr. Aaron Novick and Dr. Milton Weiner are currently studying some phases of the production of inducible enzymes. It has been demonstrated that there are two steps in the mechanism of enzyme induction: the first involves the synthesis of a transport mechanism, the so-called "permease," which concentrates the inducer inside the cell, and the second involves the synthesis of enzyme in the cell. Drs. Novick and Weiner found that the interference of the β, 3-thienylalanine was at the level of the first step, that is the synthesis of a "functional" transport system. Naturally, without inducer in the cell there could be no induction, so there was no synthesis. Recently, Monod reported similar observations using other analogs of phenylalanine.

Novelli: But it does not exclude it from acting.

La Via: No, it does not exclude the interference with active synthesis in any protein. It seems though that synthesis of proteins proceeds normally and that the analogs are incorporated into proteins but that such proteins are inactive. G. Cohen has also reported findings of this type with β, 3-thienylalanine in *Escherichia coli.*

Gell[11]: I should like to comment on Dr. Wissler's point about the relation between the cell that takes up Ag and the one that produces Ab. We have been doing a certain amount of work along the same lines as Dr. Wissler, using carbon particles as markers for cells; we are, however, working with hyperimmunized rabbits and guinea pigs, which as Dr. Wissler said, may considerably change the situation. If we load the spleen of a hyperimmunized animal with carbon particles and then produce an Arthus reaction in its skin, some carbon-containing cells come into the reaction site. That I suppose is evidence that something in the cell is responsive to the stimulus of local antigen. Moreover, if we direct carbon into the lesion, either by loading the spleen first, or by injecting carbon as the lesion is developing, so that it is preferentially taken up by the activated vascular endothelium at the site of inflammation, after 5–10 days, in a few of the plasma cells (which are produced in large numbers in active lesions in rabbits under these conditions), there is an occasional carbon deposit. This seems to me to be evidence that under such conditions a cell that has been phagocytic at one time or another is capable of turning into a plasma cell, and so presumably of producing antibody. This, as I say, occurs in hyperimmunized rabbits, a different species and a different type of immunization from those of which Dr. Wissler has spoken; but under these conditions at least it seems that the same cell may both phagocytose Ag and subsequently produce Ab.

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[10] M. F. La Via, University of Chicago.

[11] P. Gell, New York University College of Medicine.

HAHN[12]: In regard to the point raised by Dr. Novelli — use of a known inhibitor of protein synthesis at a concentration level that produces morphological and presumably biochemical evidence of this inhibition should result in a highly toxic effect of the inhibitor on the system under study. I wonder if you have any data on the toxicity of thienylalanine in your system. Correlation of the toxicity of thienylalanine with the specific effect on Ab formation might provide some indirect evidence that action on protein synthesis is involved.

WISSLER: Our study of β, 3-thienylalanine began before we tested its effect on Ab formation. This included its general effect on protein metabolism in the well-nourished adult animal as compared to the protein-deficient animal. We have always administered this substance in a diet under tube feeding conditions, so that a constant amount was being given daily throughout the experimental period and presumably with a constant amount being absorbed from the gastrointestinal tract. We gave up to about four times the usual concentration of phenylalanine in the maintenance amino acid ration; i. e., up to about 100 mg of β, 3-thienylalanine per day. This does not kill these rats, nor do they appear ill over a 10-day observation period.

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[12] F. E. Hahn, Walter Reed Army Institute of Research.

The interesting thing about this compound, as far as we are concerned, and the point that we are now working on is the fact that it does not seem to have very much effect on mitotic activity of the testes, lymph nodes, or gastrointestinal tract, but it has a rather remarkable effect on the mitotic activity in these Ab-forming cells and on the cells in transplanted neoplasms.

A great deal more study is needed to find out what role this antagonist has in the mitosis of these particular kinds of cells.

SHAPIRA[13]: I was formerly with Dr. Ditmer of Florida State, and I might mention some results that he and Dr. Crane obtained with β, 3-thienylalanine in yeast. When it is fed at a nontoxic level along with phenylalanine, he finds a very large incorporation of the C^{14}-labeled thienylalanine into the protein of the yeast. In some respects, this suggests that the inhibition may not be at the level of protein synthesis but at a higher level of organization. The biological properties of these modified proteins are under investigation.

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[13] R. Shapira, Oak Ridge National Laboratory.

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HYPERSENSITIVITY

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ONE FIGURE

One advantage of being assigned a subject such ''Hypersensitivity'' (or allergy, the terms will be used interchangeably) is that it permits one to talk about almost anything that one's conscience or discretion allows. I have chosen to speak about one small segment of the field, about the ''immediate'' or anaphylactic-type reaction, sedulously excluding any consideration of the so-called ''delayed'' types of allergy and the so-called Arthus reaction. The anaphylactic-type allergy is characterized by an antigen-antibody reaction occurring in the tissues, in which the antibody is of the circulating type. This antigen-antibody reaction leads to cellular damage that, in the typical anaphylactic reactions, is manifested by a release of histamine, serotonin, heparin, and other pharmacologically active materials. In the discussion that follows, the release of these substances will be looked upon as a sign of cellular damage and the question of the role of these substances in anaphylaxis will be ignored. Rather, attention will be focused on the manner in which the antigen-antibody reaction leads to the cellular damage.

Essentially, two hypotheses have been advanced to explain the way in which the cellular damage is evoked by the antigen-antibody reaction. The first explanation is that the antigen-antibody reaction itself, by changing in some unspecified manner the permeability of the cell, allows the release of these active substances (Dale, '52). This hypothesis will not be considered; mainly, because it presupposes more knowledge of the biochemistry of cell permeability than is presently available to us.

Rather, attention will be focused on the hypothesis that the antigen-antibody reaction activates an enzyme or series of enzymes that injure the cell (Jobling and Petersen, '14; Bronfenbrenner, '48; Rocha e Silva, '41; Ungar, '53; Burdon,'51). In discussing the evidence for this point of view, the major consideration will be given to reviewing the recent work on the enzymic mechanisms in complement action (Lepow *et al.*, '54; Lepow and Pillemer, '55; Lepow, Ratnoff, and Pillemer, '56; Lepow *et al.*, '56; Levine, '55; Becker, '55, '56a, b), the mechanism of histamine release from sensitized guinea pig lung (Mongar and Schild, '57a, b) and from rabbit platelets (Humphrey and Jaques, '55), along with related matters.

It is of course not usual to consider immune hemolysis, the lysis of a red cell by antibody against the cell and by complement, in a discussion of the allergic reaction. If, however, one defines allergy as the study concerned with the causes, nature, and consequences of the cellular and tissue damage induced by an antigen-antibody reaction, one certainly sees that in immune hemolysis there is cellular damage induced by an antigen-antibody reaction. The cellular damage in this instance is evidenced by the release of hemoglobin, whereas in anaphylaxis it is manifested by the release of such substances as histamine. There seems, therefore, no ground in logic not to look on the immune hemolysis reaction as essentially an allergic reaction (Becker, '57). This logic, it should be noted, does not involve the idea that the mechanism of immune hemolysis is necessarily the same as the mechanism of all the anaphylactic-type reactions. Yet, because the immune hemolysis reaction is, from the experimental point of view, the simplest of the anaphylactic-type reactions, an understanding of the mechanisms involved might contribute ways of looking at and into the more complex reactions. In addition, if the view of a number of workers is correct — that complement is actually involved in anaphylaxis, then the investigation of complement action might serve as more than a study of a model reaction.

The earliest notion of complement action was that an antibody to the red cell or hemolysis combined with the cell and then an entity — complement found in fresh, unheated serum — took part in the lysis of the cell. It was found, however, that complement was not a simple entity but could be divided into at least four parts or components. The four components of complement were named in the order of their discovery as the first component, the second, the third, and the fourth. For convenience, and by agreement, complement is called C' and these four components are termed C'_1, C'_2, C'_3, and C'_4. A large amount of work has gone into the definition of these components and the manner in which they act (Pillemer, '43; Heidelberger and Mayer, '48; Marrack, '55). In this respect, the work of Levine, Mayer, and their collaborators (Levine and Mayer, '54; Levine *et al.*, '54; Mayer *et al.*, '54) on the complex sequence of reactions of the components of guinea pig complement with the sensitized sheep red cell is noteworthy.

The first work to be described is that of Lepow and his collaborators (Lepow *et al.*, '54; Lepow and Pillemer, '55; Lepow, Ratnoff, and Pillemer, '56; Lepow *et al.*, '56). They observed that plasmin, a proteolytic enzyme of serum, inactivated the C'_2 and C'_4 of human complement. This inactivation occurred only in the presence of C'_1. It was postulated that C'_1 existed in serum in a proenzyme form, and that plasmin activated the precursor form of C'_1. The activated C'_1 in turn inactivated C'_2 and C'_4. Since the antigen-antibody precipitates acted in a manner paralleling that of plasmin, it was further assumed that the antigen-antibody aggregate could also convert the C'_1 precursor to a state where it was capable of destroying C'_2 and C'_4 activity.

In subsequent work, precipitates were treated in the cold with whole fresh serum and then washed. If such precipitates were added to C'_2 and C'_4 at 37°C., inactivation of C'_2 and C'_4 occurred even in the absence of C'_1. The process occurring in the cold was ascribed to the formation of an "active aggregate," one that contained activated C'_1. This had the properties of an adsorption process. The destruction of C'_2 and C'_4 by the "activated aggregate" had the properties of an enzymic reaction.

The well-washed "active aggregate" was eluted with 0.15 *M* NaCl. The concentrated eluate was capable of inactivating C'_4 and C'_2 and hydrolyzing N-acetyl-L-tyrosine methyl ester, *p*-toluenesulfonyl-L-arginine methyl ester (TAMe), and N-acetyltryptophan ethyl ester.

In further tests of the hypothesis of the proenzyme nature of C'_1, the C'_1 was partially purified from the precipitate of human serum formed at *p*H 5.5 and low ionic strength. The partially purified C'_1 was stable at *p*H 5.5 and ionic strength 0.3, but lost its hemolytic C'_1 activity when the solution was adjusted to *p*H 7.4, ionic strength 0.15. Concomitantly with the loss of C'_1 activity, the preparation became capable of destroying C'_4 and C'_2 activity, and there was a parallel appearance of an esterase hydrolyzing TAMe, and N-acetyl-L-tyrosine ethyl ester. From these experiments the authors concluded that ". . . C'_1 exists in serum as a proenzyme which is converted to an active esterase by antigen-antibody aggregates" (Lepow, Ratnoff, and Pillemer, '56).

An entirely different approach was that of Levine ('55) and of Becker ('55, '56a), who studied the inhibition of complement by diisopropylfluorophosphate (DFP). Certain phosphate esters, of which DFP is one of the most effective, will specifically inhibit esterases (Balls and Jansen, '52), and under the proper conditions only esterases. Levine showed that DFP at concentrations of 1×10^{-2} to 2×10^{-3} *M* inhibited whole guinea pig complement in the presence of sensitized red blood cells. He also showed that much lower concentrations of

DFP would inactivate sensitized sheep red blood cells containing the first and fourth components of complement. This I confirmed, and in addition made the observation that DFP would not inhibit the activity of C' unless sensitized cells were present (an observation also made by Levine). When sensitized sheep red blood cells containing C'_1 and C'_4 activity were treated with concentrations of DFP of 1×10^{-3} M to 8×10^{-5} M, inhibition of only C'_1 was noted; there was no inactivation of C'_4.

TABLE 1

Effect of 0.05 M nicotinohydroxamic acid on C′ activity of diisopropylfluorophosphate-treated $EAC'_{1,4}$[a]

MOLAR CONCENTRATION OF DFP	NICOTINOHYDROXAMIC ACID	NO NICOTINOHYDROXAMIC ACID
$\times 10^{3}$	*percentage hemolysis*	*percentage hemolysis*
9.3	56	40
4.6	76	44
0.93	92	54
0.00	100	100

[a] $EAC'_{1,4}$ are sensitized sheep cells containing C'_1 and C'_4 activity. (From Becker, '56b.)

On treatment of the DFP-treated cells containing C'_1 and C'_4 with nicotinohydroxamic acid (Wilson and Meislich, '53) a partial but clear-cut restoration of C'_1 activity was observed, as can be seen in table 1. This restoration of activity was taken as very strong evidence that the DFP inactivation of C'_1 is caused by an alkylphosphorylation. Thus the findings permitted the conclusion that C'_1 is an esterase.

The finding that DFP is capable of inhibiting the hemolytic activity of complement in the presence of sensitized cells but not in their absence was taken as indicating that C'_1 exists in serum in an inactive precursor form, a proesterase that is not capable of being inhibited by DFP. During the course of immune hemolysis, the precursor is turned into an active esterase that is capable of being inhibited by DFP. The hypothesis of the change of C'_1 from precursor enzyme to active enzyme is of course the same as that reached earlier by Lepow *et al.* ('54).

Attention was next turned to an investigation of what type of esterase C'_1 might be (Becker, '56b). Sensitized sheep red blood cells containing C'_1 and C'_4 were used as the source of enzyme and many of the amino acid esters employed by Troll *et al.* ('54) were used as possible substrates. It was shown by paper chromatography and acid production that only TAMe was regularly split by these complemented cells but not by control sensitized cells. This hydrolysis had the characteristics of an enzyme reaction.

It was possible to show, under a variety of conditions, a quantitative correlation between C'_1 activity and TAMe esterase activity of sensitized cells containing C'_1 and C'_4. When the source of sheep cells and the concentration of a single hemolysin was varied, or when the source of hemolysin was varied and the sheep cells kept constant, the C'_1 activity of the cells and the TAMe esterase activity varied correspondingly. When the amount of complement used to form the cells containing the first and fourth components was decreased, the TAMe esterase and the C'_1 activity of these cells were changed in the same characteristic way. When cells containing C'_1 and C'_4 were incubated with varying amounts of ethylenediamine tetraacetic acid (EDTA), those concentrations of EDTA that removed C'_1 activity removed TAMe esterase activity, and vice versa. When the cells were incubated with varying quantities of DFP, corresponding amounts of C'_1 and TAMe esterase activity were inactivated.

Probably the strongest single bit of evidence equating C'_1 with TAMe esterase activity was the finding that the presence of TAMe would specifically protect against the DFP inactivation of C'_1 (table 2). From this and previously cited evidence it was concluded that C'_1 is a TAMe esterase.

TABLE 2

Effect of substrate addition on DFP inhibition

	C'_1 ACTIVITY
	percentage of control
Experiment A: 5.0 × 10^{-3} *M* DFP at 21°C. overnight	
DFP alone	18
DFP + 0.01 *M* TAMe	27
DFP + 0.02 *M* TAMe	40
DFP + 0.01 *M* ATrEe [a]	18
No DFP	100
Experiment B: 1.0 × 10^{-3} *M* DFP at 37°C. for 30 minutes	
DFP alone	0
DFP + 0.02 *M* TAMe	100
DFP + 0.02 *M* TA [b]	0
NO DFP	100

[a] N-Acetyl-L-tryptophan ethyl ester. (From Becker, '56b.)

[b] Because of insolubility of tosyl arginine the actual concentration was slightly less than 0.02 *M*.

Thus the work described demonstrates that at least one of the components of complement is an enzyme. It is apparent that only a start has been made on an understanding of the nature of this enzyme and how it is involved in the over-all mechanism of complement action. On the basis of admittedly indirect evidence, I (56b) postulated that C'_1 is a proteolytic enzyme, and further speculated that one or all the other components of complement might be proteases. In support of this is the finding of Cushman *et al.* ('57) that a number of amino acid esters and peptides will quite specifically inhibit complement action and the studies of Geiger ('52a, b), Ungar *et al.* ('53), and of Plescia *et al.* ('57) showing that protease activity is associated with complement action.

As far back as 1914-15, Jobling and Petersen ('14) and Bronfenbrenner ('48) hypothesized that in anaphylaxis a proteolytic enzyme was activated and this activated enzyme caused the cellular damage. The similarity of this concept to the work on complement just described is evident. Since that time a number of other workers (Rocha e Silva, '41; Ungar, '53; Burdon, '51) have evoked this or similar explanations of the allergic reaction. Others have, however, denied even the fact of protease activation during the course of the antigen-antibody reaction (Jemski *et al.,* '53), although the weight of the evidence seems to indicate that protease activation does occur (Ungar, '53; Burdon, '51; Humphrey and Jaques, '55; Geiger, '52b). However, even some of those that accept the fact of protease activation doubt its relevance to the allergic reaction (Mongar and Schild, '57b; Geiger, '52b; Dale, '29). Part of the difficulty is purely verbal, arising from the belief that there is only one serum protease. For example, it has been the fashion of many workers to speak of ''fibrinolysin'' or ''plasminogen'' activation by the antigen-antibody reaction with assumption being made explicitly or implicitly that the enzyme (or enzymes) so activated is the same as the enzyme activated by streptokinase. Actually, in no work has any evidence been presented that ''plasminogen,'' the streptokinase-activable enzyme, is activated by an antigen-antibody reaction, and there *is* evidence to the contrary. Thus the argument that streptokinase injection (Johnson *et al.,* '57) leading to active plasmin formation does not lead to anaphylactoid symptoms or that ''fibrinolysin'' does not release histamine (McIntire, '56) at best merely shows that this enzyme, under the specified conditions, is not responsible for the anaphylactic reaction. It allows no inference to be drawn as to the possibility of other proteolytic enzymes under other conditions being involved.

But the difficulty is more than verbal. It has been shown that the proteolytic enzyme, trypsin, can release histamine (Rocha e Silva, '39) and, as pointed out previously, the weight of evidence is that the action of the antigen-antibody aggregation as well as other substances whose action leads to histamine release is associated with proteolytic activity. But the question remains whether, under these circumstances, the proteolytic activation and the histamine release are causally connected or whether the same process that leads to histamine release leads to proteolytic activation, or whether they are merely parallel phenomena. Certainly, the evidence presently available does not permit a decision among these alternatives.

Turning from this cursory consideration of the role of proteolytic enzymes in anaphylactic shock, one can ask whether complement plays a role in the more complex anaphylactic reactions similar to that described for immune hemolysis.

Several authors (Witebsky and Neter, '35; Bier and Seiler, '36) have suggested that complement is involved in reversed passive Forssman shock, the shock produced when serum containing the Forssman antibody is injected into animals containing the Forssman antigen. This suggestion is most plausible when it is realized that there is an exact analogy between this type of shock and immune hemolysis. When the Forssman antibody is injected it combines directly with the antigen, which is an integral part of the cells of the vascular endothelium as well as other sites, exactly as hemolysin combines with the antigen on the red cell. Moreover, the Forssman antibody can damage the sheep red cell when it combines in the presence of complement with the Forssman antigen on the red cell. In addition, Hyde ('32) showed that a strain of guinea pigs deficient in complement activity would not give reversed passive Forssman shock. Guinea pigs with normal complement levels, of course, give this type of shock.

Friedberger and Hartoch ('09) first expressed the idea that complement might be responsible for the more typical anaphylactic shock. This hypothesis was subsequently more or less discarded but has been revived by a number of investigators including Schwab *et al.* ('50), Lange *et al.* ('51), Ungar *et al.* ('53), Lepow *et al.* ('54), and still later, Bier *et al.* ('55). The hypothesis of Friedberger and Hartoch was essentially based on the finding that during anaphylactic shock a fall of complement occurs. This observation has since been confirmed by numerous workers (Rice, '55; Stavitsky *et al.*, '49). The question, however, remains unanswered as to whether this represents a direct implication of C′ in anaphylaxis or the fall is merely an expression of the fixation of complement occurring because of the antigen-antibody reaction and having nothing to do with the shock.

A number of authors have pointed out that there is a general correlation between the ability of an antigen-antibody reaction to fix complement and to cause anaphylactic shock, e.g., horse, sheep, and cow antibody generally fix guinea pig complement either very poorly or not at all. Similarly, antisera from these species are generally not capable of giving anaphylactic shock in the guinea pig. This correlation is not absolute: horse antibody against hemocyanin fixes guinea pig complement very well, but will not give anaphylactic shock (Follensby and Hooker, '44). Thus the ability to fix complement might, at best, be a necessary but not sufficient condition for a given antibody to produce anaphylactic shock.

Probably the most direct evidence so far published of the possible participation of C′ in anaphylaxis is that of Bier *et al.* ('55). In their experiments, the intravenous injection of one antigen-antibody system into a rat reduced the C′ level. When the complement titer had been reduced to about 5 $C'H_{50}$ units, the passive cutaneous anaphylaxis reaction using a completely unrelated immune system was abolished. This work is being pursued further by Doctors A. Osler and Z. Ovary and I shall leave their results to be described by Doctor Osler in the open discussion.

Humphrey and Jaques ('55) pointed out the similarity between histamine release from rabbit platelets by an antigen-antibody reaction and C′ fixation. They showed that the presence of plasma or serum, and of calcium ions was essential for the reaction. Heating of the plasma at 56°C. for ½ hour caused a great loss of the ability of the antigen-antibody reaction to cause histamine release. From the finding of a parallelism between histamine release and protease activation, they suggested the "... following sequence of events: antigen-antibody combination $\xrightarrow{Ca^{++}}$ fixation of some component(s) of complement, followed by activation of protease." How well this hypothesis fits into the work of Haining ('56) and of Barbaro ('57) on the ability of antigen-antibody precipitates to release histamine in the same system, only further work will tell.

Even if C′ is involved in anaphylaxis, it is possible that the ratios of activities of the various components are different or that not all the components that are required in immune hemolysis are required in anaphylaxis. This might serve as an explanation as to why Hyde was able to show that his complement-deficient guinea pigs were as susceptible to the usual type of anaphylaxis as were normal pigs (Hyde, '32). Since the strain of complement-deficient guinea pigs died out and is no longer available, these observations are presently unreproducible; it is therefore difficult to know what weight to attach to them. Nevertheless, they do point up the need for caution in coming to any conclusion that C′ is involved in the anaphylactic reaction.

More direct insight into the biochemical mechanisms of the anaphylactic reaction is furnished by the very exciting work of Mongar and Schild ('57a, b) on the release of histamine from guinea pig lung slices by means of an antigen-antibody reaction. These authors showed that the *in vitro* release of histamine from lung slices of guinea pigs actively sensitized to egg albumin, on the addition of egg albumin, is affected by a large number of factors. It is inhibited by a number of metabolic inhibitors such as iodoacetate, chlorimercuribenzoate, cyanide, 2,4-dinitrophenol, by lack of oxygen, by antipyretics such as phenylbutazone, amidopyrine, antipyrine, salicylates, and related compounds such as phenol. In all these cases the inhibition is reversible. Mongar and Schild further showed that the inhibition was not caused by the inhibition of the antigen-antibody reaction but of a subsequent, and they felt, probably an enzymic reaction.

In a study of the effect of temperature on histamine release in the same system, Mongar and Schild ('57a, b) showed that there was almost no histamine release at 20°C., it rose to a maximum between 35½° and 42½° and fell abruptly beyond this temperature. They concluded that the heat inactivation was not caused by either denaturation of antibody or displacement of antibody from the tissue site.

If antigen was added to sensitized tissue at 17°C. and the mixture kept there for 15 minutes, no histamine was released; but if at the end of this time, the reaction mixture was brought to 37°C. then histamine release was normal. If, however, the antigen was left in contact with antigen at 17° C. for one hour, very little histamine was released on bringing the tissue to 37° C.

The scheme the authors used to account for their observations is seen in figure 1. Here it is seen that the antigen-antibody reaction leads to an activation of an enzyme system, which in turn catalyzes reactions leading to histamine release. This activated enzyme becomes rapidly inactivated. Inhibitors act at different sites.

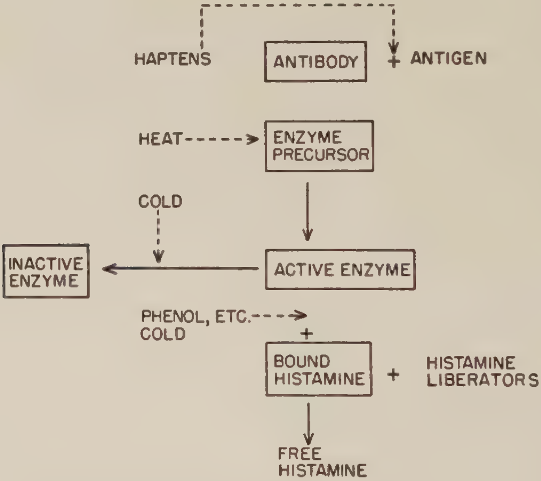

Fig. 1 The mechanism of anaphylaxis according to Mongar and Schild. (From Mongar and Schild, '57a.)

On the basis of these experiments, the authors discard the proteolytic theory of histamine release, pointing out that " . . . temperatures above 45° C. inactivate the anaphylactic mechanism but activate the proteolytic mechanism" (Mongar and Schild, '57a). They also believe that the factor in normal tissue that is required in anaphylaxis is not identical with serum complement, mainly on the basis that the temperature of inactivation of complement is distinctly higher than that for cellular histamine release.

Actually, though differences obviously exist, there are striking similarities between the findings of Mongar and Schild on histamine release and those of Levine *et al.* ('54) on the mechanism of immune hemolysis. Mongar and Schild showed that in histamine release there is a step that can occur at low temperatures and also a step requiring higher temperatures (whether these are two different reactions is not known), and at least one of the factors concerned is very heat labile. Levine, Mayer, and their coworkers (Levine and Mayer, '54; Levine *et al.*, '54; Mayer *et al.*, '54) demonstrated with guinea pig complement that part of the immune hemolysis reaction, the fixation onto the cell of C'_1 C'_2, and C'_4 activities goes very well in the cold, but that for the complete reaction to occur involving the activity of C'_3 the temperature must be raised. They have also shown that the activity of C'_2 on the sensitized cell is very temperature sensitive, C'_2 having a half life at 37°C. of only about 10 minutes, although in whole serum C'_2 is more stable.

SUMMARY

In regard to immune hemolysis, we have the sequence of reactions partially worked out, the identification of one of the components of the reaction as an enzyme, and some information as to the nature of the enzyme. In typical anaphylaxis we have some suggestive, but far from conclusive, evidence that complement may be involved. Concerning the *in vitro* analog of typical anaphylaxis, release of histamine from sensitized guinea pig lung or from rabbit platelets by an antigen-antibody reaction, we have very suggestive evidence that enzymic activation is part of the mechanism and that certain similarities as well as differences exist between this process and that of immune hemolysis. We also have an array of substances in circulating blood such as anaphylatoxin or serotoxin, studied by Rocha e Silva and Aronson ('52) and the "permeability factor" studied by Miles and Wilhelm ('55) and other substances that have been shown to have the ability to release histamine. The relation of these last substances to anaphylaxis is still a matter of speculation, but it is considered probable that here, too, they are activated enzymes or enzyme activation plays a role in their formation.

We are unable to say at present that the underlying mechanism of such reactions is the same for every type of reaction occurring in every species. We can say, at the most, that in all the various types investigated, enzyme activation by an antigen-antibody reaction seems to be part at least of the mediating mechanism. This, on one hand, furnishes a great hope that we shall ultimately understand the biochemical mechanism of the various anaphylactic-type reactions, since we now have powerful techniques available for handling enzymic reactions. On the other hand, any similarity between one type of anaphylactic reaction and another might be attributable to the presence of this general mechanism of enzyme activation rather than an identity of the processes or the specific enzymes involved. The need for this caution is exemplified by the history of the conceptions about the relations of the coagulation of blood, the activation of plasminogen, and the hemolytic action of complement. At one time or another it has been considered that any one of the processes mentioned was supposed to contain elements present in at least one of the other processes. In fact, there is a common feature in all three processes — the central mechanism in each is the activation of a specific enzyme, and each enzyme is a TAMe esterase. In addition, the general complexity of the process is the same. Yet each of the TAMe esterases involved — thrombin, plasmin, and C'_1 is a separate and distinct enzyme and there is no present evidence for interconnection of any of the three processes except the report of Lepow *et al.* ('54) that plasmin can activate C'_1. An analogous situation might well turn out to be that concerning the various types of the anaphylactic reaction. The hope nevertheless remains that there might be an identity of underlying mediating mechanism extending through the various types and species. This can be known with certainty only when the mechanisms of the various types have been worked out in detail.

OPEN DISCUSSION

OSLER[1]: We have attempted to determine whether one type of anaphylactic reaction, namely, passive cutaneous anaphylaxis (PCA), involves the participation of complement. In essence, we have manipulated the levels of circulating complement in albino rats and have shown a correlation between the hemolytic activity of the sera of these animals and their reactivity to PCA.

Our findings may be summarized as follows: Rats rendered deficient in complement by an unrelated immune system are quite refractory to the dermal anaphylactic response. There is a close parallel in the time sequence of these two events. Both the complement levels and skin reactivity diminish and return at the same time. Furthermore, it has been possible to restore the skin reactivity of such complement-depleted animals by the injection of fresh serum, or serum treated so as to retain the third complement component activity. Serum treated with zymosan for the preferential destruction of the third component of complement does not restore the anaphylactic response. In normal rats, the intensity of the skin response can be enhanced by injection of hemolytically active guinea pig or rat serum, or preparations manifesting third component activity.

[1] A. G. Osler, Johns Hopkins University.

The ability of different antigen-antibdy systems to elicit this skin response seems to be directly correlated with the potency of these immune reagents to fix complement either in the test tube or in the body of the albino rat. In the experiment mentioned by Dr. Becker, it should be noted that horse antiprotein systems may fix complement *in vivo,* but fail to elicit skin responses in guinea pigs because of the inability of the equine antibody to be fixed to the skin sites involved in the allergic reaction. Although there is no doubt that many factors influence the immediate type of allergic reaction, our evidence is suggestive, but not conclusive, that complement participates in the mediation of passive cutaneous anaphylaxis in the albino rat.

Rapport[2]: Dr. Becker, can you reconcile this mechanism with anaphylaxis of the Schultz-Dale type, where complement can hardly be involved; or should we for the time being consider individual target organs as offering quite distinct mechanisms?

Becker: Actually, this brings up a very old controversy, the controversy between the cellular and the humoral mediation of the anaphylactic reaction. One can possibly reconcile this if one assumes that there is an absorption of these components into the target cells, or that extremely small amounts of complement still exist after washing. I think this is one of the things that has to be investigated in detail. I might point out the possibility that under certain circumstances one could have a humoral mechanism, under other circumstances a largely cellular one. This might reconcile the need for having a well-defined latent period under certain conditions of anaphylaxis with the fact that under certain other conditions this latent period can be reduced or almost abolished.

The question of whether the so-called incomplete antibody or coprecipitating antibody fixes complement is of some interest, since Kabat showed that this antibody is just as capable as precipitating antibody in passively sensitizing guinea pigs.

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[2] M. M. Rapport, Sloan-Kettering Institute for Cancer Research.

WAKSMAN[3]: About 7 years ago Dr. Benacerraf, who was working with Dr. Kabat, gave me a sample of nonprecipitating antibody. Its ability to fix complement was closely comparable to that of the samples of precipitating antibody that I was studying at that time.

OSLER: We found that some serum samples (sent to us by Dr. Pappenheimer) that had skin-sensitizing properties also fixed complement *in vitro*. Furthermore, rabbit antibody ribonuclease fixed complement in the test tube and elicited PCA reactions. However, when the same antibody was retested with an acetylated derivative of the enzyme, little, if any, complement was fixed in the test tube and PCA reactions were not demonstrable.

Definitive conclusions concerning the complement-fixing potency of a serum cannot be drawn from results obtained with the conventional type of test performed with about five 50% units of complement. Experience in our laboratory with several different immune systems indicates the fixation of complement may be observed in tests carried out with 100, but not with 5, units of complement.

PRESSMAN[4]: Speaking of the implication of complement in cellular damage — in the cases of human glomerulonephritis, Lange has reported a drop in complement during the acute phase, supposedly caused by reaction of antibody with tissue, and resulting in tissue damage. Also, further implication of complement in damage mediated by nephrotoxic serum in animals is perhaps shown by the difference in nephrotoxic effect of avian and mammalian antirat kidney sera reported by several investigators. In this case, it appears that there is a delayed nephrotoxic effect of avian serum and an immediate effect of the mammalian serum. This we have suggested is perhaps because the mammalian serum fixes complement in the kidney and causes damage whereas the avian serum is not known to fix complement and does not cause immediate damage.

[3] B. H. Waksman, Harvard University.

[4] D. Pressman, Roswell Park Memorial Institute.

Dr. Beatrice Seegal, at Columbia, however, observed no immediate effect with antikidney serum prepared in fowls.

Rapport: I should just like to reiterate this point by calling attention to certain cytotoxic effects of antisera on cultures of cells in *in vitro* studies. It is reported that in some cases complement is necessary for observation of the cytotoxic effects, in others, it does not seem to play any part whatever.

We must bear in mind that although complement may exert some influence in many systems, it does not appear to be essential. It is pretty hard to visualize the mediation of complement on an isolated strip of guinea pig intestine in a water bath.

Becker: I am glad that Dr. Rapport saw fit to reiterate the point I made in the paper — that we have to consider each system and each species as "of its own kind," and to investigate each of these situations in detail before we can conclude that any general over-all mechanism operates.

Waksman: Dr. Becker is being too modest in answering Dr. Rapport's question. There are, of course, many different kinds of cell damage. In the absence of complement, antibody against a red cell has a definite effect on the red cell, which we can observe but which is not the type of red cell lysis that releases hemoglobin. The particular process that Dr. Becker discussed is a quite specific effect involving the release by particular cells of histamine, serotonin, heparin, and so on. There is a good deal of reason to believe that these cells are not simply the muscle cells of the intestine or the uterine strip but mast cells that are present in these tissues and that quite definitely will release these compounds under these circumstances.

There seems to me to be a very real difficulty inherent in the data discussed by Dr. Becker. As he has pointed out, a number of metabolic inhibitors will prevent histamine release from sensitized guinea pig lung. These are presumably inhibitors that affect respiratory metabolism or other fundamental cellular processes. It is difficult to put together the series of findings involving complement and proteolytic activity, which one would in general visualize as affecting a cell membrane or perhaps the binding of histamine within the cell, and these other findings that clearly implicate the actual metabolism of the cell itself.

Becker: Discussing the last point first: It has been shown that histamine is contained in the mitochondria in a form that does not normally leak out. It is at least possible that the postulated activated enzyme releases histamine through an effect on the metabolism of the cell, allowing histamine to be released from the mitochondria and out of the cell. In other words, where we have so little information we can always erect a hypothesis that will ''save the phenomenon.'' But the preceding remarks point up the fact that the release of histamine is apparently a much more complex process than the release of hemoglobin, and even if the over-all mechanism is the same or similar (and I spent a great deal of my talk stressing how, even where there is evidence pointing in this direction, the evidence might be misleading), we would not expect to obtain necessarily a one-to-one correspondence between the two processes.

With regard to the question of the participation of mast cells, in a lecture before the Academy of Allergy (Becker, '57), I pointed out how simple it would make things to consider anaphylaxis a manifestation of mast cell lysis in the same way as red cell lysis is a manifestation of immune hemolysis. I also pointed out how wary one must be of erecting a general theory on this concept. For example, it is generally considered that in the rabbit the platelets are the source of the serotonin and histamine released, and it is probably the injury to the platelets that is the fundamental cause of the anaphylactic injury in this species.

Similarly Perry gave 48-80 in sufficiently large doses to rats to induce a breakup of the mast cells, with no apparent effect on the release of histamine by the passive cutaneous anaphylaxis reaction. This work, although not conclusive, suggests the very real possibility that in this species also the mast cells might not be involved in the anaphylactic reaction.

Since we are quite ignorant of the actual mechanism of the activation process, we can only consider the possibilities in terms of known reactions. Drs. Lepow and Ratnoff have some evidence that the activation process is an autocatalytic phenomenon analogous to the trypsinogen-trypsin transformations. How the antigen-antibody reaction initiates this supposed autocatalytic reaction is not known. The antigen-antibody aggregate may have certain of the properties of the proteolytic enzyme analogous to the action of trypsin initiating the chymotrypsinogen-chymotrypsin conversion. There is absolutely no evidence for this possibility. It is also possible that the activation process has a tendency to go spontaneously and is only prevented from doing so by an inhibitor. There is some evidence for this from the work of Lepow and Ratnoff. The antigen-antibody reaction might then bind the inhibitor. There is no evidence for this. One could also assume that the precursor form of C'_1 is analogous to the trypsin-soybean trypsin inhibitor complex and that the antigen-antibody reaction removes the inhibitor. This is another possibility for which there is no evidence.

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IMMUNOLOGY OF BONE MARROW TRANSPLANTATION

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EIGHT FIGURES

Previous discussions in this symposium reveal that concerted efforts in bone marrow transplantation are relatively new. Much is yet to be done in unraveling the complex puzzle that exists in this field. I shall discuss only three of the many problems: the role of the recognition factor of an antibody-producing cell, the mechanism of the delayed foreign bone marrow reaction, and the antigen involved in the delayed foreign bone marrow reaction.

I. RECOGNITION FACTOR OF AN ANTIBODY-PRODUCING CELL

One of the fundamental problems concerning the mechanism of antibody production is how an antibody-producing cell can recognize that a substance is foreign and just how foreign it is. Based on the accepted definition of antigen and antibody, it is implicit that the term "foreign" is *qualitative* rather than quantitative. Our results on the recovery of the immune mechanism after varying doses of X radiation to the total body, lethal irradiation and treatment with bone marrow, and transplantation of bone marrow after X irradiation strongly suggest that the ability of the antibody-producing cell to *recognize* that a substance is foreign has quantitative implications (Makinodan, '56; Makinodan *et al.*, '56, '57; Makinodan and Shekarchi, '57; Gengozian and Makinodan, '56).

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[1] Operated by Union Carbide Nuclear Company for the U. S. Atomic Energy Commission.

Using induction period, rate of appearance of free circulating antibodies, mean peak titer, and mean total titer as indices of antibody production, we found that normal mice respond to rat red blood cell (RBC) antigen equally as well as to sheep RBC antigen (Makinodan *et al.*, '56, '57; Makinodan and Shekarchi, '57) (see figure 1). Immune response studies performed 1, 15, and 30 days after irradiation, however, showed that the response to sheep RBC was better than to rat RBC. A typical example with 475 r is shown in figure 2 (Makinodan *et al.*, '57). A hypothesis (I) drawn from these data would be: There could exist either multiple types of antibody-producing cells, or an antibody-producing cell, with the ability to recognize varying degrees of antigenicity; i.e., homologous,[2] closely related heterologous, and distantly re-

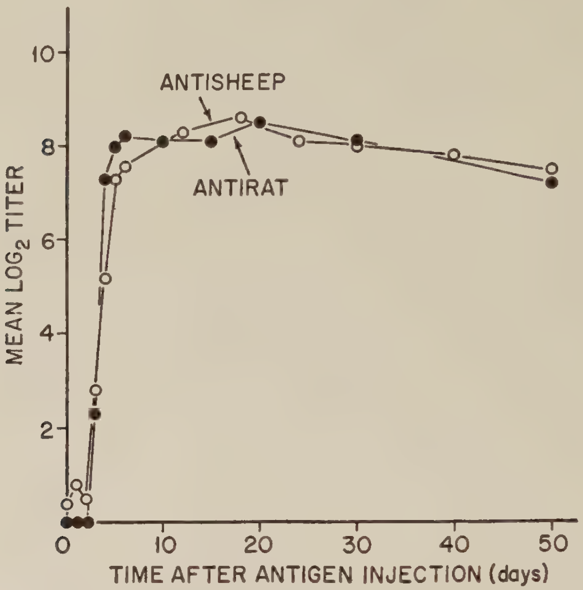

Fig. 1 Sheep and rat agglutinin production by normal 12-week-old (C3H × 101) F_1 mice.

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[2] Isologous denotes intrastrain differences within a species, homologous denotes interstrain differences within a species, and heterologous denotes interspecies differences.

lated heterologous antigens, and the degree of destruction by X rays would increase in the order of mention.

Corroborative data can be found in the various bone marrow transplantation studies. Lindsley *et al.* ('55) and Ford *et al.* ('56) presented direct evidence that homologous bone marrow (HBM) injected into irradiated recipients does transplant.

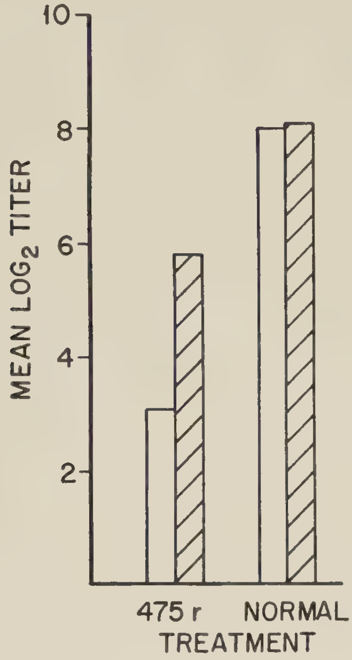

Fig. 2 Effect of type of antigen on the immune mechanism (mean total $\log_2$ titer of 1, 15, and 30 days after treatment); least significant difference (LSD, 5%) is 0.51. Open columns, antirat response; shaded columns, antisheep response; 180 mice per group.

Others have shown it by indirect evidence (Main and Prehn, '55; Vos *et al.*, '56; Mitchison, '56; van Bekkum *et al.*, '56; Merwin and Congdon, '56). Direct evidence that rat bone marrow (RBM) does transplant when injected into lethally irradiated mice has been presented independently from several laboratories (Makinodan, '56; Ford *et al.*, '56; Vos *et al.*, '56; Nowell *et al.*, '56). Congdon *et al.* ('52) found indirect evidence of a few successful transplants of guinea pig bone marrow in lethally irradiated mice; distantly related heterologous bone marrow cells never transplanted in these mice. Lorenz and Congdon ('53; Congdon, unpublished data) reported that dog and monkey bone marrow would not transplant in mice exposed to lethal doses of radiation. In our experiments, chicken (marrow from as many as five tibias) and rabbit (as much as 300 mg of neonatal rabbit marrow) marrow failed to transplant in 950-r (C3H × 101) F_1 mice.

It can be argued that the failure of distantly related heterologous bone marrow to transplant in 950-r mice is caused by the cytotoxic effect of naturally occurring antibodies. In validity tests of this assumption (Makinodan *et al.*, '57), we found mouse agglutinins with an average $\log_2$ titer of 1–2 in pooled normal rabbit sera (RS). Similarly, normal mouse sera contained rabbit agglutinins with an average $\log_2$ titer of 1–2. Since this reciprocal relation held in the rabbit-to-mouse transplant, isologous bone marrow (IBM) cells were suspended in pooled rabbit sera (RS) and, after 10 minutes' incubation at room temperature, were resuspended in Tyrode's solution and injected into mice exposed to 950 r. Simultaneous runs were made with 950-r, 950 r-IBM, and 950 r-RS mice.[3] Deaths in the 950-r and 950 r-RS mice followed the expected pattern; all died within 2 weeks. The 950 r-IBM and 950 r-IBMRS mice were still alive 60 days after treatment. These results indicate that, although aggregation of mouse bone marrow cells did occur in RS, no cytotoxic effect was evident under the experimental condition, since these treated cells were still capable of protecting lethally irradiated mice.

Based on these transplantation data, hypothesis I seems more acceptable. Tests of this hypothesis were therefore carried out. 2-Mercaptoethylguanidine hydrobromide (MEG) has proved to have about a twofold X-ray dose-reducing effect, in terms of the rate of recovery of the immune mechanism (Makinodan *et al.*, '57). Transplantation of rat bone marrow did take place in 950-r mice but *not* in MEG-950 r mice; HBM transplanted in *both* the 950-r and the MEG-950 r mice. In the second validity test, evidence for transplantation of guinea pig bone marrow in irradiated mice was determined by the alkaline phosphatase technique. Guinea pig bone marrow failed to transplant in *950*-r mice but did transplant in three of seven *1150*-r mice. These results would be expected on the basis of hypothesis I.

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[3] The classes, or groups, of mice used in these experiments will be designated as follows. The order of the symbols and numbers indicates the order of administration. 950-r, 950 r-IBM, 950 r-RBM, 950 r-RS, and 950 r-IBMRS. In the last group, the IBM had been treated with RS. The 30-day LD_{100} for this strain of mice is ~875 r.

II. MECHANISM OF THE DELAYED FOREIGN BONE MARROW REACTION

In lethally irradiated mice that receive foreign bone marrow, such as from normal rats (RBM) or normal mice of another strain (HBM), radiation death is prevented; but many die subsequently of a delayed reaction (Makinodan, '56; Gengozian and Makinodan, '56; Barnes and Loutit, '54; Cole *et al.*, '55). Lethally irradiated mice given IBM do not show this delayed death pattern (fig. 3). If the change in body weight is used as an index, it can be seen that mice tend to recover from radiation effects within 2 weeks, but begin to lose weight about the third week after treatment (figs. 4, 5). The critical period appears about the fifth or sixth week; recovery then follows. As shown in figure 5, essentially three types of change in body weight were observed among the 950 r-RBM mice, although all carried rat marrow transplant. Mice showing no weight change were least frequently observed. Electrophoretic analysis of serum samples obtained from 950 r-RBM mice at varying intervals showed protein changes analogous to the changes in body weight. During the most critical period of the delayed foreign bone marrow reaction (i.e., 5–6 weeks after treatment), the decrease in the albumin/globulin ratio was caused by an absolute increase in γ-globulin and a decrease in albumin (fig. 6). As shown in table 1, these 950 r-RBM mice responded weakly to sheep RBC but not at all to rat or mouse RBC (Gengozian and Makinodan, '56). Evidence of transplantation of bone marrow has been discussed in detail. On the basis of these observations, we concluded that the delayed death pattern is attributable to an *in vivo* antigen-antibody reaction. If this explanation is accepted as the underlying cause of the deleterious effect on the host, three explanations

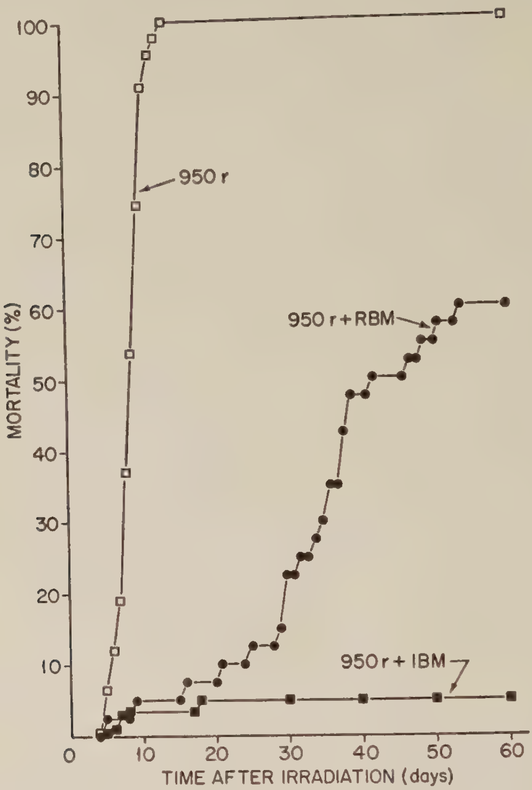

Fig. 3 Effect of bone marrow on mortality of lethally irradiated (950 r) (C3H × 101)F_1 mice. (From Gengozian and Makinodan, '56).

are possible: (1) antibody-producing cells present in the transplanted bone marrow are reacting against the host, (2) the radiosensitive antibody-producing cells of the host have recovered from the radiation injury and are reacting against the transplanted proliferating foreign bone marrow, or (3) a combination of (1) and (2).

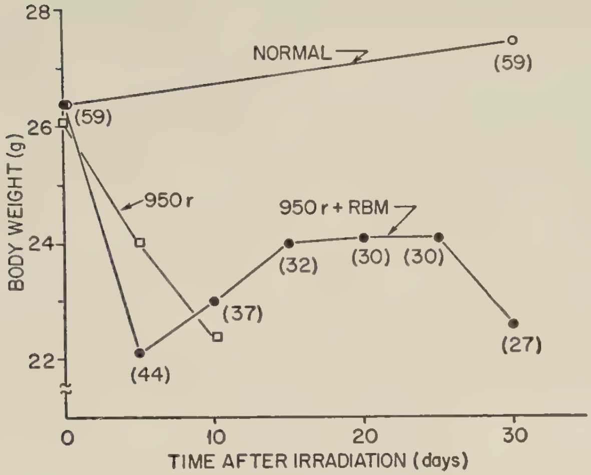

Fig. 4 Body weight changes of mice lethally irradiated (950 r) and protected with rat bone marrow. Numbers in parentheses indicate numbers of animals.

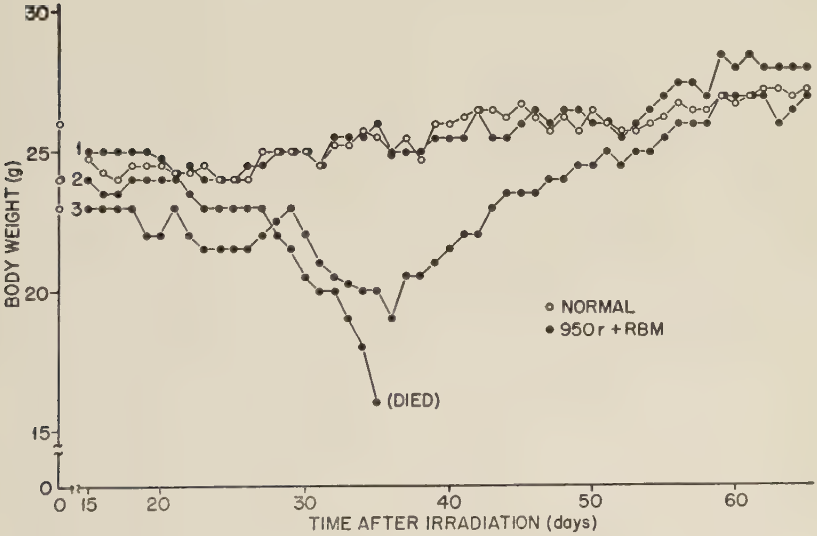

Fig. 5 Three types of change in body weight of lethally irradiated (950 r) mice given rat bone marrow. (From Makinodan, '56.)

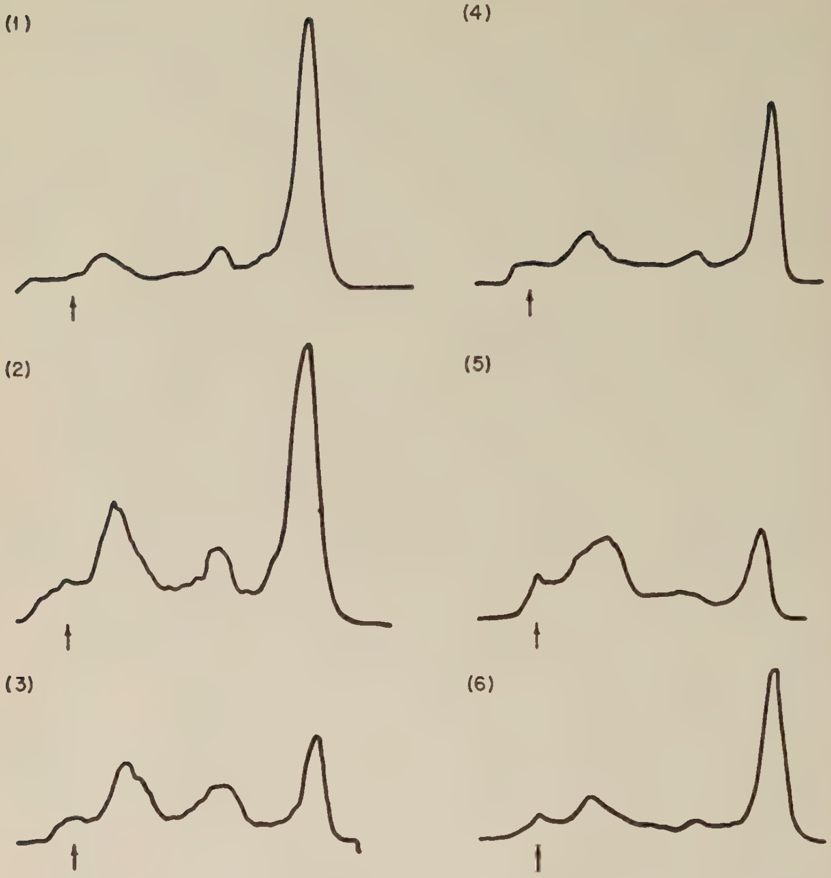

Fig. 6 Representative electrophoretic patterns of serum samples of (C3H × 101) F_1 mice obtained at varying intervals after 950 r and rat bone marrow treatment (veronal buffer, *p*H 8.6, ionic strength 0.075, constant current 5 ma, 20 hours at room temperature).

Treatment	*mg of Protein N/ml*	*Days after treatment*
(1) None	9.60	..
(2) 950 r	9.95	8
(3) 950 r-RBM	5.00	8
(4) 950 r-RBM	6.70	24
(5) 950 r-RBM	8.30	40
(6) 950 r-RBM	7.25	60

It was reasoned that if the transplant were producing antibodies against the host, globulin of the transplant type should be present in high concentration. Specific mouse antirat and rat antimouse sera were used after preliminary experience with rabbit antisera. (Although rabbit antisera gave higher titers, the cross reaction between rat and mouse sera was too high. Cross absorption of the rabbit antisera could have been carried out under a careful condition; but we felt that since there exist at least six immunochemically distinct antigens of unknown combining ratios in a given serum, the "specific" antiserum after absorption may contain soluble antigen-antibody complexes of varying nature that could interfere with the final precision of the test.) The specific antisera employed were capable of detecting as little as 12 μg of protein nitrogen/ml. Serum samples from 950 r-RBM mice were obtained at intervals of 7–65 days after treatment. In no case were we able to detect any rat protein by the interfacial and the double serum agar diffusion tests. We therefore concluded that if rat protein were present in these serum samples, it must be in concentration lower than 12 μg of protein/ml. A representative result is shown in figure 7.

TABLE 1

Agglutinin production in treated and untreated (C3H × 101)F_1 mice

(Mean peak antibody titer $\log_2$)

RBC ANTIGEN	NORMAL CONTROL	RBM CONTROL	950 r-RBM		
			Day 15[a]	Day 30	Day 60
Rat	8.7	8.5	0.0	0.0	. .
Sheep	8.5	8.5	0.3	2.6	2.9
Mouse	0.0	. .	. .	. .	0.0

[a] Day after protection on which immune response studies began.

Pertinent data were also obtained from immune response studies. The 950 r-IBM mice responded to sheep RBC antigen better than to rat RBC antigen at intervals of 1–30 days after treatment (Makinodan *et al.*, '56). A representative finding is shown in figure 8. We reasoned that if the bone marrow

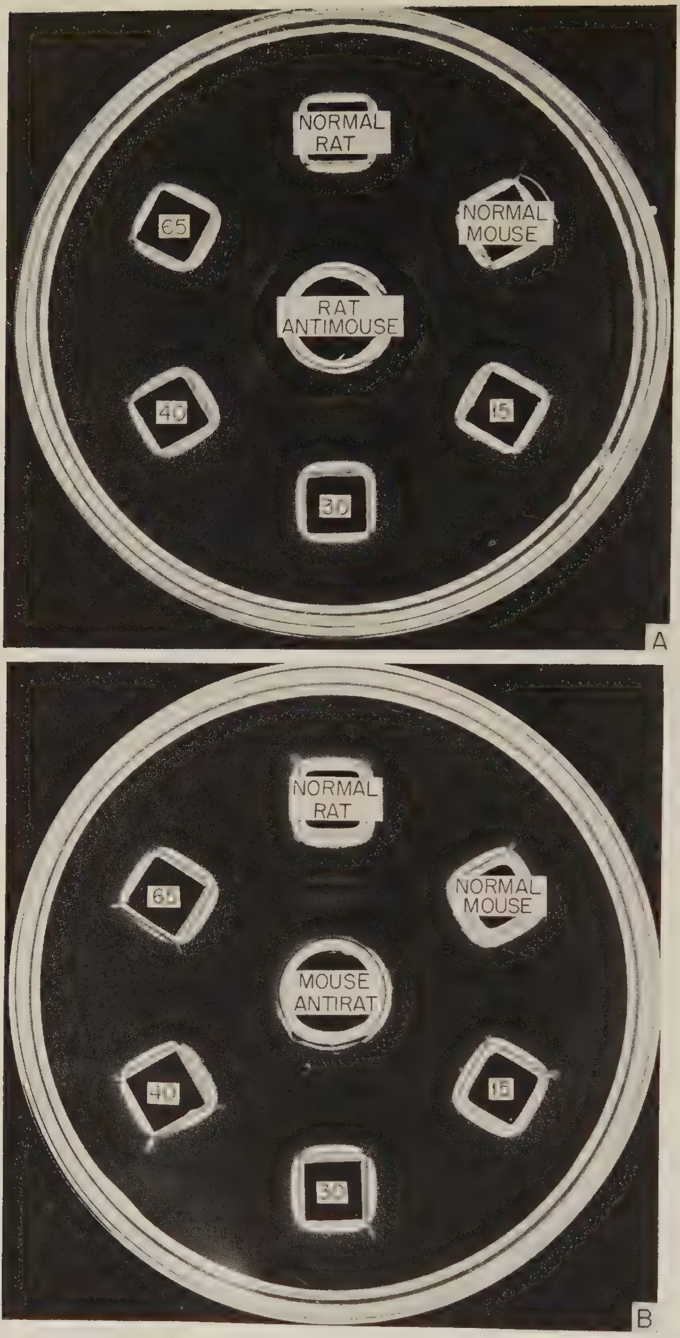

Fig. 7 Agar diffusion patterns of serum samples of (C3H × 101) F_1 mice obtained at varying intervals after 950 r and rat bone marrow treatment (photographed after 6 days' incubation). A, rat antimouse serum; B, mouse antirat serum. Numerals indicate number of days after treatment.

contributed significant amounts of antibody-producing cells, the rat agglutinin production should have been comparable to the sheep agglutinin production, since the transplanted cells were not affected by the radiation. It was interesting that when 12 × 10^6 normal isologous nucleated *spleen* cells were injected simultaneously with rat RBC antigen into 950-r mice, circulating antibodies were detectable in 5 days. On the other hand, no circulating antibodies were detected in 950-r mice injected simultaneously with 12 × 10^6 normal isologous nucleated *bone marrow* cells and rat RBC antigen. This amount of bone marrow was at least ten times the optimum amount necessary to protect 950-r mice. From sheep hemolysin production studies, Smith and Ruth ('56) concluded that injection of IBM cells shortly after sublethal irradiation was ineffective in hastening the recovery of the immune response of mice.

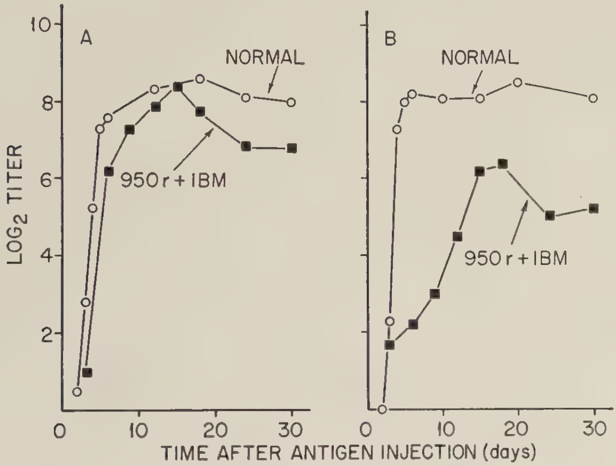

Fig. 8 Sheep and rat agglutinin production by (C3H × 101)F_1 mice 30 days after 950 r and isologous bone marrow treatment. (From Makinodan, Gengozian, and Congdon, '56.)

These findings led to the conclusion that the second explanation for the mechanism of delayed foreign bone marrow reaction (shown schematically) is the most plausible; i.e., lethal irradiation has caused a temporary impairment of the immune mechanism of the host, and this permitted the transplantation of closely related foreign bone marrow. In time, however, there is a gradual recovery of the host's antibody-producing cells, and this will react against the proliferating foreign transplant. This explanation becomes hypothesis II.

$$(\mathrm{Ag})_x\,(\mathrm{Ab})_y \rightarrow \text{delayed reaction}$$

where

$$\text{foreign bone marrow} \rightarrow \mathrm{Ag} \text{ and}$$
$$\text{host} \rightarrow \mathrm{Ab}$$

when x and y are units of combining ratios.

Corollary A: X-ray dose $= k_1$ host $= k_2$ (Ab synthesis)

Corollary B: Bone marrow dose $= k_3$ (Ag).

A and B are corollaries of this idea. If this idea is incorrect, then (1) the *degree* of foreign bone marrow reaction should be *independent* of the X-ray dose, provided that the dose is the minimum that will permit transplantation of the foreign marrow; (2) the *time* of the foreign bone marrow reaction should be *independent* of the X-ray dose; and (3) the mortality with a given X-ray dose should increase with the increase in the bone marrow dose. Tables 2 and 3 summarize the results of validity tests. It can be seen in table 2 that the degree of foreign bone marrow reaction is *dependent* on the X-ray dose. As shown in column 4 of this table, with a constant bone marrow dose, all mice were killed by 710 r of X rays, in contrast to 58, 22, and 25% mortality with 800, 950, and 1150 r, respectively. This result is in agreement with hypothesis II—that the lower the X-ray dose, the less the destruction of the immune mechanism of the host, and thus the faster the recovery of the immune mechanism leading to a more vigorous antigen-antibody reaction. Columns 4, 5, and 6 show that the time of foreign bone marrow reaction, as indicated by the mortality, is also *dependent* on the X ray dose. Mice receiving less than 710 r showed no change in death pattern after 30 days. At 400 r, no RBM transplantation occurred. Survivors of 500 r- and 600 r-RBM groups showed no transplants. The greatest mortality in the 800 r-RBM group was in the first 30 days, with 58% death. During the 30- to 60-day period, only 15% of the mice died, followed by 4% death during the 60- to 150-day period. In the 950 r-RBM group, mortality was highest in the 30- to 60-day period—37% death, in contrast to 22% death during the first 30 days and 4% during the 60- to 150-day period. This observation is also in accord with hypothesis II. Finally, if antibody-producing cells were derived from the transplant and were reacting against the host, one would expect to find an increase in death with increase in bone marrow dose. The data in table 3 refute this expectation. From these results, we concluded that, if the transplants were

TABLE 2

Mortality of X-irradiated (C3H × 101)F_1 mice receiving no treatment, isologous bone marrow, or rat bone marrow

X-RAY DOSE	CUMULATIVE PERCENTAGE MORTALITY AFTER INDICATED TREATMENT				
	None	IBM (12×10^6 cells)	RBM (140×10^6 cells)		
	Day 30	Day 30	Day 30	Day 60	Day 150
r					
400	0	0	0	0	0
500	0	0	15	15	15
600	5	0	50	50	50
710	30	0	100	..	..
800	79	0	58	73	77
950	100	5	22	59	63
1150	100	25	25	40	65

TABLE 3

Effect of varying the amount of nucleated rat bone marrow cells on the mortality of (C3H × 101)F_1 mice

NUCLEATED RAT BONE MARROW CELLS	X-RAY DOSE (r) ⟶ 800	950
	30-day mortality	
1×10^6	%	%
0	79	100
25	95	50
45	100	
75	85	25
140	58	22

also reacting against the host, the magnitude was of an order not detectable by our methods.

It is still not known what mechanisms are involved when an animal undergoes a transition from a state of *immunity* to a state of *tolerance,* or *paralysis,* as indicated by 950 r-RBM mice that recovered fully from the foreign bone marrow reaction. Investigation along this line is now being undertaken in our laboratory.

III. ANTIGEN IN THE DELAYED FOREIGN BONE MARROW REACTION

Our numerous attempts to detect complete or incomplete antibodies against cellular antigens from these 950 r-RBM mice failed (Makinodan, '56). We therefore deduced that the antigen involved in this foreign bone marrow reaction could be nuclear instead of cytoplasmic. Four groups of 6-week-old mice were sensitized to rat RBC, WBC, whole blood, and bone marrow cells, respectively. At 12 weeks of age, these mice were given 950 r and again injected with rat bone marrow. The results are tabulated in table 4. Mice presensitized with RBC did possess circulating rat agglutinins at 12 weeks of age. The 10% mortality in this group is comparable to that for nonsensitized 950 r-RBM mice. In contrast, mice presensitized with nucleated cells and treated with 950 r-RBM 6 weeks later showed 100% mortality. These results suggest that the nuclear antigen, rather than the cytoplasmic antigen, is involved in the foreign bone marrow reaction.

TABLE 4

Effect of type of presensitizing rat antigen on the survival of (C3H × 101)F_1 mice subsequently given 950 r and rat bone marrow at 12 weeks of age

MICE PRESENSITIZED WHEN 6 WEEKS OLD	
Presensitizing rat antigen	30-Day mortality
1 × 10^6 cells	%
Red blood cells	10
Whole blood	70
White blood cells	100
Bone marrow cells	100

Conclusion. The role of the recognition factor of an antibody-producing cell and its relative sensitivity to X rays has a great implication, not only to radiation immunology but to general biology. Obviously, much work is still required to elucidate this problem.

From an immunologist's point of view, one of the most fascinating phenomena of the bone marrow transplantation studies is the delayed foreign bone marrow reaction. The task of circumventing or subduing the reaction is a formidable one, considering the large number of immunochemically distinct antigens existing in a given bone marrow suspension.

Finally, our preliminary studies seem to indicate that the nuclear membrane or the nuclear material, or both, are mainly responsible for the delayed foreign bone marrow reaction. Should the nuclear material be found to be the chief agent involved in this delayed reaction, the problem then becomes ever more challenging since the antigenic material is chemically impure and its solubility in aqueous medium is limited.

ACKNOWLEDGMENTS

Most of the recent data presented in this paper were obtained in close cooperation with Drs. N. Gengozian and Isabel C. Shekarchi of this Laboratory, to whom the author expresses his gratitude.

OPEN DISCUSSION

ANDERSON:[4] The cell physiology group at Oak Ridge is engaged in a rather long-range work in fractionation of cells in tissues; and some of these techniques are applicable to fractionation of suspension cells. The technique we are exploring at the outset is using a centrifuge tube containing high-density material in the form of a gradient, in this case bovine serum albumin, increasing in concentration on down the cell. The material to be separated is introduced as a thin layer on top.

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[4] N. G. Anderson, Oak Ridge National Laboratory.

After 5-minute centrifugation at 800 rpm, the platelets and cell debris are at the top, two layers of cells are seen farther down the tube, and then clumps, and some large cells that hit the bottom. The upper cell layer is mostly red blood cells and the smaller cells. They appear not in two types, as histologists might wish them to, but in two ranges of size and density. We are hoping to continue this work with other techniques, including isopyknic centrifugation. For the present purposes, we wanted to know whether the cells would survive this particular treatment, and it turns out that one can get protection with material from the general region of the second cell layer. I think that Dr. Makinodan might say something about the nature of the survivors. All experiments were done with rat bone marrow, and tests were done in mice.

MAKINODAN: By injecting the various *cuts* into 950-r mice, we obtained three types of survivors: (1) those with transplants of RBC and granulocyte precursors, (2) those with transplant of granulocyte precursors, and (3) those with only a temporary transplant of granulocyte precursors. It thus seems to indicate that a minimum requirement of temporary transplantation of granulocyte precursors is vitally important in preventing death of lethally irradiated mice, and that transplantation of RBC precursors is a concomitant phenomenon. No test for transplantation of platelet precursors was carried out.

VAN BEKKUM[5]: We have studied a number of irradiated mice that have been treated with rat bone marrow for about a year, and have followed the nature of the erythrocytes and the granulocytes in the peripheral blood. Briefly, three different patterns were observed after the initial 2-month period necessary for the complete replacement of mouse erythrocytes by rat erythrocytes: (1) Animals in which all the erythrocytes and the granulocytes remain of the rat type for the duration of the observation period (more than 1 year); (2) animals that subsequently get rid of all foreign erythrocytes and granulocytes ("total reversals," a term proposed by Dr. Loutit); (3) animals that contain a mixture of mouse and rat cells for long periods of time.

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[5] D. W. van Bekkum, Medical Biological Laboratory of the National Defense Research Council, T. N. O., The Netherlands.

Dr. Makinodan, have you any information about the relation between the condition of the graft and the delayed mortality? So far we have not been able to find any clear relation.

MAKINODAN: At the outset I would like to state that we have observed the first two types of survivors but not those with a mixture of mouse and rat cells for a long period. It is agreed that lethally irradiated mice treated with foreign bone marrow undergo a delayed reaction, some of which will recover in time. At present, these survivors can be considered to be in a state of tolerance, or paralysis, or both. Obviously, there are at least two basic problems that must be elucidated before the status of these survivors can be predicted with reasonable certainly: (1) mechanism of the transition from a state of immunity to a stage of tolerance, or paralysis, or both, and (2) establishment of the status of these survivors. If these survivors were in a state of threshold paralysis, fluctuation of the mixture of mouse and rat cells would be expected, but if they were in a state of tolerance, or extreme paralysis, no fluctuation would be expected.

VAN BEKKUM: You are quite right. Indeed, in most of the cases that show a mixed population for longer periods of time, we find fluctuations. It is only in some very rare cases that we find a constant proportion of foreign cells maintained.

LOUTIT[6]: I was very pleased that Dr. Makinodan started by making the point about the difference between host and graft—that the homologous tissue is probably more closely related than rat, which is more closely related than guinea pig, and so on down the scale. When it comes to the vexing question of whether it is the host reacting against the graft or the graft reacting against the host, or both, then the bulk of your experience is with the mouse-rat mixture. The bulk of our experience is with the mouse-mouse mixture. So quite probably both observations are correct. In all probability,

[6] J. F. Loutit, Radiobiological Research Unit, AERE, England.

both reactions occur, but in one, one predominates and in the other the other predominates. We have heard a lot at this meeting about the multiplicity of antibody and the multiplicity of reactions. The important thing is to decide what reaction we regard as the most significant. On the agglutinin reaction, I cannot remember whether it was you or Dr. Congdon who pointed out it is not necessarily a cytotoxic reaction, and it is quite possible that rat cells are surviving in this chimera in the presence of antirat agglutinin. I would regard the critical reaction to look at in such a mouse-rat chimera as another tissue transplantation; one of Dr. van Bekkum's slides of a skin transplant was a very elegant demonstration. In fact, his particular animal, I think, was entirely rat as far as its bone marrow was concerned.

I have a slide of a similar graft of rat skin on one of these mouse-rat chimeras. I don't know the exact constitution of this particular animal; but his mate, that died about a couple of weeks previously with a perfectly good skin graft, according to Dr. Ford had mixed mouse and rat cells in its bone marrow and lymphoid tissue. This mouse was grafted about a fortnight after the establishment of the chimerism. It was grafted with rat-tail skin, so that it has not grown a nice beard as van Bekkum's has done, but it does show the white patch in perfectly good shape 75 days from the time of grafting. When I left it was in equally good condition at over 100 days after the graft.

Cole[7]: Some of our experiments are in direct agreement with some of the statements that Dr. Makinodan made; namely, we have been unable to show transplantation of rabbit or dog bone marrow in lethally irradiated mice, using the alkaline-phosphatase technique as a marker.

Then, we have done preliminary studies, similar to Dr. Makinodan's on presence of agglutinins in irradiated mice injected with rat spleen and bone marrow cells. Using death as an endpoint, we injected rat bone marrow cells into irradiated mice, and at the same time, or the same day, injected mouse spleen cells. Under these circumstances, the death pattern is accelerated; i.e., the presence of the injected isologous spleen cells apparently accelerates the time of death, thereby nullifying the rat bone marrow effect. Alkaline phosphatase-positive cells (rat cells) are already absent by 8 days. In most of these treated animals, the time of death corresponds to that of nontreated control animals. But if we give rat bone marrow and mouse (isologous) bone marrow at the same time, the animal survives indefinitely.

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[7] L. J. Cole, U. S. Naval Radiological Defense Laboratory.

Makinodan: With regard to the skin graft experiment, successful rat skin takes on lethally irradiated mouse treated with rat bone marrow and does not invalidate our hypothesis. On the contrary, this would be expected, regardless of whether the animal is in, or is approaching, the state of tolerance, or paralysis. In such experiments, we cannot disregard the general physiological status, of which the immune status is a part, in relation to the type and dose of antigen (bone marrow and skin cell antigens).

Koller[8]: It seems that the results of his experiments convinced Dr. Makinodan that the so-called secondary disease and delayed death should be attributed to the host reaction against the donor graft. I believe that the reaction of the graft against the recipient host may also be important. If we compare the number of casualties in semi-isologous chimeras, when the recipient is of hybrid constitution, with that when the recipient is one of the parental strain, the importance of the graft becomes clear.

X-rayed host	*Donor*	*Survival*
F_1(A × C57BL)	A	Low number of 30-day survivors
F_1(A × C57BL)	C57BL	
A	F_1(A × C57BL)	Higher number of 30-day survivors
C57BL	F_1(A × C57BL)	

Our data, though very meager, suggest that F_1 is better as donor than as host. It is very likely when the parental strain is used as donor, that the donor cells react with the antigens of hybrid host and produce antibodies against the other parental strain involved in the cross. Dr. Amos of Roswell Park, Buffalo, has been experimenting with chimeras of hybrid constitution from the serological aspect, and we hope that with his technique and collaboration, the antibodies produced by the donor graft can be demonstrated.

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[8] P. C. Koller, Chester Beatty Research Institute.

MAKINODAN: What are the 30-day LD_{50} and LD_{100} for the four strains of mice?

LOUTIT: I can provide only some of the data, and I am afraid it will not satisfy you. The LD_{50} for the parent strains in our laboratory is 700–750 r but we have too few of the F_1 to do a run. I would guess, however, that the dose that Dr. Koller used is well above the LD_{50} and, as he says, above the LD_{100}.

UPTON[9]: I find these results interesting because, unless I am confused in my memory, Dr. van Bekkum found that the parental material went into the F_1 recipient with better success than the F_1 into the parent. Perhaps the answer to this question may lie in the nature of the cells injected and their number.

Again I should like to inquire if, in Dr. Koller's experiments, marrow was used and about how many cells, as opposed to the possibility that spleen might have been used. In Dr. van Bekkum's experiment, marrow was used exclusively.

KOLLER: The technique of Dr. Loutit (he has very extensive data) is using one animal as the donor. The 1-ml saline suspension of bone marrow cells is divided among all five of the X-rayed recipients, i.e., two-fifths of femur bone marrow goes into one mouse. The number of cells injected varies from 2×10^6 to 4×10^6.

UPTON: No spleen?

KOLLER: No spleen was given in these cases.

VAN BEKKUM: I am sorry but I should like to clear this confusion. We have estimated the number of bone marrow cells required to give 30-day survival, using C57BL, CBA, and

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[9] A. C. Upton, Oak Ridge National Laboratory.

(CBA × C57BL)F_1 mice in every possible host-donor combination.

Dr. Upton is quite right. We found, indeed, that we need many more F_1 cells to protect the parent strain mice than we need parent strain cells to protect the F_1 mice. In the former case the number of cells required equals the number of homologous cells and in the latter combination the number of cells is approximately equal to the number required in the case of effective treatment with isologous cells. However, these are all 30-day survival studies.

With regard to the delayed mortality, we used only the animals that had been treated with what we consider optimal number of cells; that is, in the case of isologous cells: about 1–2 million; in the case of homologous and heterologous cells, 25–50 million cells. The surprising observation was made that, after administering F_1 cells to the irradiated parent strain mice or, reversely, after giving parent strain cells to irradiated F_1 mice, practically no delayed mortality occurred.

CONGDON: I think that fits in with our experience, too. The delayed mortality is certainly not what you would expect from the homologous type experiment—giving F_1 bone marrow to the parent.

I was inclined to explain the result on the basis of the recognition factor, thinking that the F_1 would be less foreign than the other parent. If we exchange bone marrow between the two parents of the hybrid, the homologous reaction develops but the hybrid into either parent does not give the homologous reaction. I wonder whether the hybrid is closer antigenically to either parent than one parent to another. The recognition would follow Makinodan's scheme.

BRUNER[10]: As one who must bring up a practical question, how shall we regard the human transplantation? If we could get an opinion I would appreciate it very much. Man to man, assuming the usual broad grouping and cross-matching, is this an example of isologous, homologous, or heterologous?

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[10] H. D. Bruner, USAEC, Division of Biology and Medicine.

Koller: Apart from the case of monozygotic or identical twins, all transplantations in man should be considered to be homologous; i.e., the donor cells or tissues will be reacted against and destroyed by the host. Yet in certain circumstances in which the gravely ill patient's immune mechanism is depressed, a homograft of bone marrow may offer a new method of therapy.

Two attempts of marrow transfusions were carried out by one of our colleagues, Dr. H. E. M. Kay of Royal Marsden Hospital, with the primary aim to try out the method of administration. Fresh ribs were obtained from chest operations performed for nonmalignant conditions. By use of two pairs of Adson's forceps, the marrow can be squeezed out of the end of the rib like toothpaste out of a tube; 2–3 ml is thus collected into an equal quantity of acid citrate dextrose mixture.

The first case receiving marrow transfusion was a patient who had had about 4000 r to a large retroperitoneal tumor. At completion of treatment, a site was selected in the right posterior iliac crest, where there was shown to be almost complete aplasia. The whole donor sample was injected there, but some difficulty was experienced in the actual injection, which could only be made extremely slowly. The object was to reexamine the site of injection and to do studies with Fe^{59} to test for selection uptake at the site. Unfortunately, this was refused by the patient. Blood counts, however, were done and, since the patient was group A and donor group O, Ashby agglutination tests were performed to detect foreign cells. No such cells could be demonstrated, and routine blood counts showed no effect that could be ascribed to the marrow implant.

The other case was a patient with bone marrow aplasia caused by an overdose with Myleran. She was gravely ill at the time of transfusion, when the major portion of donor marrow was injected intravenously and a small quantity was inserted into one iliac crest. Death occurred 2 days later. A portion of bone taken from the region of the implant was sectioned. Dr. Kay is not convinced that there was any sign of cellular repopulation of the marrow.

These cases are mentioned to show that homologous bone marrow transplantation technique is possible in human patients. The results of such transplantations would be of great interest to study.

QUASTLER[11]: What do you imagine the nature of the recognition factor to be? There is a large difference in a few patches whereas there are equal differences in many patches. It could be tested.

MAKINODAN: I don't know; at the moment, this is a hypothetical property. I might add that if irradiation is a reversion of the immune status to some embryonic state, then one of the ways of testing this hypothesis is studying the development of the immune mechanism in terms of the various types of antigen in relation to age.

FOX[12]: I should like to suggest an idea that might help reconcile two seemingly unrelated problems—(1) why the introduced marrow cells settle in the bone marrow of the host, and (2) the correlation of success of transplantation with the genetic relation between donor and host. The idea is that of Paul Weiss, who suggests that during embryological development, surface configurations of cells play an important role in their final arrangement with respect to each other, such that cells with complementary surface configurations remain adjacent to each other while an absence of complementarity results in continued movement. Is it possible that the possession of a specific ''bone marrow configuration'' might serve to fix isologous and homologous cells in the marrow, whereas deviations from this configuration might result in the continued wandering and ultimate loss of sufficiently remote heterologous cells?

Congdon[13]: This work involved giving massive doses of isologous marrow to normal mice without being able to count any difference in cellularity in the host marrow. But in mice irradiated 3 days earlier, Paul Urso found increased cellularity at the end of 20 minutes. How this works with foreign marrow may apply to what you are saying. Maybe the marrow cells do not fit correctly and cannot get through to the marrow spaces.

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[12] A. S. Fox, Michigan State University.

[13] C. C. Congdon, Oak Ridge National Laboratory.

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